EXHIBIT B

US006284478B1

(12) **United States Patent**

Heller et al.

(54) **SUBCUTANEOUS GLUCOSE ELECTRODE**

(75) Inventors: **Adam Heller; Michael V. Pishko**, both of Austin, TX (US)

(73) Assignee: **E. Heller & Company**, Alameda, CA (US)

(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/767,110**

(22) Filed: **Dec. 4, 1996**

Related U.S. Application Data

(63) Continuation of application No. 08/299,526, filed on Sep. 1, 1994, now Pat. No. 5,593,852, which is a continuation-in-part of application No. 08/161,682, filed on Dec. 2, 1993, now Pat. No. 5,356,786.

(51) **Int. Cl.^{7}** **C12Q 1/54**; C12Q 1/00; C12Q 1/26; C12Q 1/28

(52) **U.S. Cl.** **435/14**; 435/4; 435/25; 435/28; 435/817; 435/287; 436/63; 436/149; 204/403

(58) **Field of Search** 435/4, 14, 25, 435/28, 817, 287; 436/63, 149; 204/403

(56) **References Cited**

U.S. PATENT DOCUMENTS

Re. 32,947	6/1989	Dormer et al.	435/14
3,260,656	7/1966	Ross, Jr.	435/14
3,653,841	4/1972	Klein	435/14
3,719,564	3/1973	Lilly, Jr. et al.	435/14
3,776,832	12/1973	Oswin et al.	435/14
3,837,339	9/1974	Aisenberg et al.	435/14
3,926,760	12/1975	Allen et al.	435/14
3,972,320	8/1976	Kalman	435/14

(10) **Patent No.:** **US 6,284,478 B1**

(45) **Date of Patent:** *****Sep. 4, 2001**

3,979,274	9/1976	Newman	435/14
4,008,717	2/1977	Kowarski	435/14

(List continued on next page.)

FOREIGN PATENT DOCUMENTS

227 029 A3	9/1985	(DD) .
29 03 216	8/1979	(DE) .
3934299	10/1990	(DE) .
44 01 400 A1	7/1995	(DE) .
0 010 375 A1	4/1980	(EP) .

(List continued on next page.)

OTHER PUBLICATIONS

Abruña, H. D. et al., "Rectifying Interfaces Using Two–Layer Films of Electrochemically Polymerized Vinylpyridine and Vinylbipyridine Complexes of Ruthenium and Iron on Electrodes," *J. Am. Chem. Soc.*, 103(1):1–5 (Jan. 14, 1981).

Albery, W. J. et al., "Amperometric enzyme electrodes. Part II. Conducting salts as electrode materials for the oxidation of glucose oxidase," *J. Electroanal. Chem. Interfacial Electrochem.*, 194(2)(1 page–13 Abstract only)(1985).

(List continued on next page.)

Primary Examiner—Louise N. Leary
(74) *Attorney, Agent, or Firm*—Merchant & Gould P.C.

(57) **ABSTRACT**

A small diameter flexible electrode designed for subcutaneous in vivo amperometric monitoring of glucose is described. The electrode is designed to allow "one-point" in vivo calibration, i.e., to have zero output current at zero glucose concentration, even in the presence of other electroreactive species of serum or blood. The electrode is preferably three or four-layered, with the layers serially deposited within a recess upon the tip of a polyamide insulated gold wire. A first glucose concentration-to-current transducing layer is overcoated with an electrically insulating and glucose flux limiting layer (second layer) on which, optionally, an immobilized interference-eliminating horseradish peroxidase based film is deposited (third layer). An outer (fourth) layer is biocompatible.

74 Claims, 10 Drawing Sheets
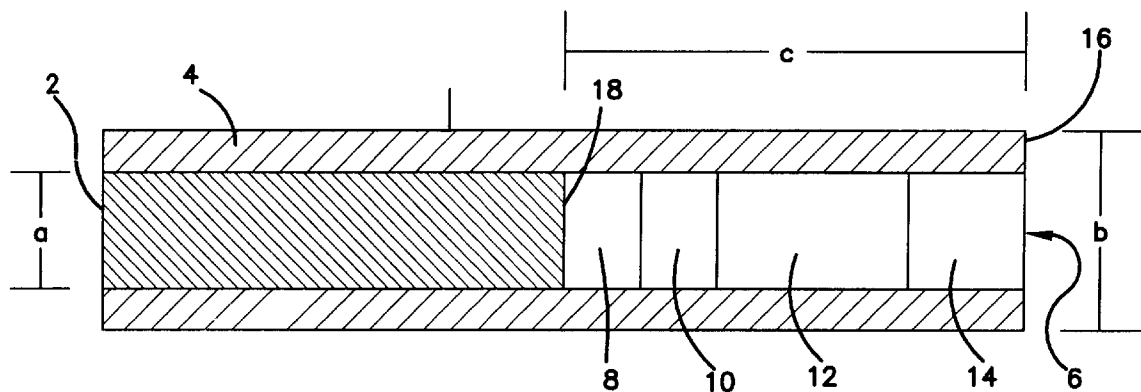
US 6,284,478 B1

Page 2

U.S. PATENT DOCUMENTS

4,016,866	4/1977	Lawton	435/14
4,055,175	10/1977	Clemens et al.	435/14
4,059,406	11/1977	Fleet	435/14
4,076,596	2/1978	Connery et al.	435/14
4,098,574	7/1978	Dappen	435/14
4,100,048	7/1978	Pompei et al.	435/14
4,151,845	5/1979	Clemens	435/14
4,168,205	9/1979	Danninger et al.	435/14
4,172,770	10/1979	Semersky et al.	435/14
4,178,916	12/1979	McNamara	435/14
4,206,755	6/1980	Klein	435/14
4,224,125	9/1980	Nakamura et al.	435/14
4,240,438	12/1980	Updike et al.	435/14
4,247,297	1/1981	Berti et al.	435/14
4,340,458	7/1982	Lerner et al.	435/14
4,352,960	10/1982	Dormer et al.	435/14
4,356,074	10/1982	Johnson	435/14
4,365,637	12/1982	Johnson	435/14
4,366,033	12/1982	Richter et al.	435/14
4,375,399	3/1983	Havas et al.	435/14
4,384,586	5/1983	Christiansen	435/14
4,390,621	6/1983	Bauer	435/14
4,401,122	8/1983	Clark, Jr.	435/14
4,404,066	9/1983	Johnson	435/14
4,418,148	11/1983	Oberhardt	435/14
4,427,770	1/1984	Chen et al.	435/14
4,431,004	2/1984	Bessman et al.	435/14
4,436,094	3/1984	Cerami	435/14
4,440,175	4/1984	Wilkins	435/14
4,450,842	5/1984	Zick et al.	435/14
4,458,686	7/1984	Clark, Jr.	435/14
4,461,691	7/1984	Frank	435/14
4,469,110	9/1984	Slama	435/14
4,477,314	10/1984	Richter et al.	435/14
4,484,987	11/1984	Gough	435/14
4,522,690	6/1985	Venkatasetty	435/14
4,524,114	6/1985	Samuels et al.	435/14
4,526,661	7/1985	Steckhan et al.	435/14
4,534,356	8/1985	Papadakis	435/14
4,538,616	9/1985	Rogoff	435/14
4,543,955	10/1985	Schroeppel	435/14
4,545,382	10/1985	Higgins et al.	435/14
4,552,840	11/1985	Riffer	435/14
4,560,534	12/1985	Kung et al.	435/14
4,571,292	2/1986	Liu et al.	435/14
4,573,994	3/1986	Fischell et al.	435/14
4,581,336	4/1986	Malloy et al.	435/14
4,595,011	6/1986	Phillips	435/14
4,619,754	10/1986	Niki et al.	435/14
4,627,445	12/1986	Garcia et al.	435/14
4,627,908	12/1986	Miller	435/14
4,633,878	1/1987	Bombardieri	435/14
4,637,403	1/1987	Garcia et al.	435/14
4,650,547	3/1987	Gough	435/14
4,654,197	3/1987	Lilja et al.	435/14
4,655,880	4/1987	Liu	435/14
4,655,885	4/1987	Hill et al.	435/14
4,671,288	6/1987	Gough	435/14
4,679,562	7/1987	Luksha	435/14
4,680,268	7/1987	Clark, Jr.	435/14
4,682,602	7/1987	Prohaska	435/14
4,684,537	8/1987	Graetzel et al.	435/14
4,685,463	8/1987	Williams	435/14
4,703,756	11/1987	Gough et al.	435/14
4,711,245	12/1987	Higgins et al.	435/14
4,717,673	1/1988	Wrighton et al.	435/14
4,721,601	1/1988	Wrighton et al.	435/14
4,721,677	1/1988	Clark, Jr.	435/14
4,726,378	2/1988	Kaplan	435/14
4,726,716	2/1988	McGuire	435/14
4,757,022	7/1988	Shults et al.	435/14
4,758,323	7/1988	Davis et al.	435/14
4,759,371	7/1988	Franetzki	435/14
4,759,828	7/1988	Young et al.	435/14
4,764,416	8/1988	Ueyama et al.	435/14
4,776,944	10/1988	Janata et al.	435/14
4,777,953	10/1988	Ash et al.	435/4
4,781,798	11/1988	Gough	435/14
4,784,736	11/1988	Lonsdale et al.	435/14
4,795,707	1/1989	Niiyama et al.	435/14
4,796,634	1/1989	Huntsman et al.	435/14
4,805,624	2/1989	Yao et al.	435/14
4,813,424	3/1989	Wilkins	435/14
4,815,469	3/1989	Cohen et al.	435/14
4,820,399	4/1989	Senda et al.	435/14
4,822,337	4/1989	Newhouse et al.	435/14
4,830,959	5/1989	McNeil et al.	435/14
4,832,797	5/1989	Vadgama et al.	435/14
4,840,893	6/1989	Hill et al.	435/14
4,848,351	7/1989	Finch	435/14
4,854,322	8/1989	Ash et al	435/4
4,871,351	10/1989	Feingold	435/14
4,871,440	10/1989	Nagata et al.	435/14
4,874,500	10/1989	Madou et al.	435/14
4,890,620	1/1990	Gough	435/14
4,894,137	1/1990	Takizawa et al.	435/14
4,897,162	1/1990	Lewandowski et al.	435/14
4,897,173	1/1990	Nankai et al.	435/14
4,909,908	3/1990	Ross et al.	435/14
4,911,794	3/1990	Parce et al.	435/14
4,917,800	4/1990	Lonsdale et al.	435/14
4,919,141	4/1990	Zier et al.	435/14
4,919,767	4/1990	Vadgama et al.	435/14
4,923,586	5/1990	Katayama et al.	435/14
4,927,516	5/1990	Yamaguchi et al.	435/14
4,934,369	6/1990	Maxwell	435/14
4,935,105	6/1990	Churchouse	435/14
4,935,345	6/1990	Guilbeau et al.	435/14
4,938,860	7/1990	Wogoman	435/14
4,944,299	7/1990	Silvian	435/14
4,950,378	8/1990	Nagata	435/14
4,953,552	9/1990	DeMarzo	435/14
4,954,129	9/1990	Giuliani et al.	435/14
4,969,468	11/1990	Byers et al.	435/14
4,970,145	11/1990	Bennetto et al.	435/14
4,974,929	12/1990	Curry	435/14
4,986,271	1/1991	Wilkins	435/14
4,994,167	2/1991	Shults et al.	435/14
5,001,054	3/1991	Wagner	435/14
5,002,054	3/1991	Ash et al.	435/4
5,058,592	10/1991	Whisler	435/14
5,070,535	12/1991	Hochmair et al.	435/14
5,082,550	1/1992	Rishpon et al.	435/14
5,082,786	1/1992	Nakamoto	435/14
5,089,112	2/1992	Skotheim et al.	435/14
5,095,904	3/1992	Seligman et al.	435/14
5,101,814	4/1992	Palti	435/14
5,106,365	4/1992	Hernandez	435/4
5,108,564	4/1992	Szuminsky et al.	435/14
5,109,850	5/1992	Blanco et al.	435/14
5,120,420	6/1992	Nankai et al.	435/14
5,126,034	6/1992	Carter et al.	435/14
5,133,856	7/1992	Yamaguchi et al.	435/14
5,135,003	8/1992	Souma	435/14
5,141,868	8/1992	Shanks et al.	435/14
5,161,532	11/1992	Joseph	435/14
5,165,407	11/1992	Wilson et al.	435/14
5,174,291	12/1992	Schoonen et al.	435/14
5,190,041	3/1993	Palti	435/14
5,192,416	3/1993	Wang et al.	435/14
5,198,367	3/1993	Aizawa et al.	435/14
5,202,261	4/1993	Musho et al.	435/14

US 6,284,478 B1
Page 3

5,205,920	4/1993	Oyama et al.	435/14
5,208,154	5/1993	Weaver et al.	435/14
5,209,229	5/1993	Gilli	435/14
5,217,595	6/1993	Smith et al.	435/14
5,229,282	7/1993	Yoshioka et al.	435/14
5,250,439	10/1993	Musho et al.	435/14
5,262,035	11/1993	Gregg et al.	435/14
5,262,305	11/1993	Heller et al.	435/14
5,264,103	11/1993	Yoshioka et al.	435/14
5,264,104	11/1993	Gregg et al.	435/14
5,264,106	11/1993	McAleer et al.	435/14
5,271,815	12/1993	Wong	435/14
5,279,294	1/1994	Anderson et al.	435/14
5,286,362	2/1994	Hoenes et al.	435/14
5,286,364	2/1994	Yacynych et al.	435/14
5,288,636	2/1994	Pollmann et al.	435/14
5,293,546	3/1994	Tadros et al.	435/14
5,320,098	6/1994	Davidson	435/14
5,320,725	6/1994	Gregg et al.	435/14
5,322,063	6/1994	Allen et al.	435/14
5,337,747	8/1994	Neftel	435/14
5,352,348	10/1994	Young et al.	435/14
5,356,786	10/1994	Heller et al.	435/14
5,368,028	11/1994	Palti	435/14
5,372,133	12/1994	Hogen Esch	435/14
5,376,251	12/1994	Kaneko et al.	435/14
5,378,628	1/1995	Grätzel et al.	435/14
5,387,327	2/1995	Khan	435/14
5,390,671	2/1995	Lord et al.	435/14
5,391,250	2/1995	Cheney, II et al.	435/14
5,395,504	3/1995	Saurer et al.	435/14
5,411,647	5/1995	Johnson et al.	435/14
5,437,999	8/1995	Diebold et al.	435/14
5,462,645	10/1995	Albery et al.	435/4
5,469,846	11/1995	Khan	435/14
5,494,562	2/1996	Maley et al.	435/14
5,496,453	3/1996	Uenoyama et al.	435/14
5,497,772	3/1996	Schulman et al.	435/14
5,531,878	7/1996	Vadgama et al.	435/14
5,545,191	8/1996	Mann et al.	435/14
5,560,357	10/1996	Faupel et al.	435/14
5,565,085	10/1996	Ikeda et al.	435/14
5,567,302	10/1996	Song et al.	435/14
5,568,806	10/1996	Cheney, II et al.	435/14
5,569,186	10/1996	Lord et al.	435/14
5,582,184	12/1996	Erickson et al.	435/14
5,582,697	12/1996	Ikeda et al.	435/14
5,582,698	12/1996	Flaherty et al.	435/14
5,586,553	12/1996	Halili et al.	435/14
5,589,326	12/1996	Deng et al.	435/14
5,593,852	1/1997	Heller et al.	435/14
5,596,150	1/1997	Arndt et al.	435/14
5,617,851	4/1997	Lipkovker	435/14
5,628,890	5/1997	Carter et al.	435/14
5,651,869	7/1997	Yoshioka et al.	435/14
5,660,163	8/1997	Schulman et al.	435/14
5,670,031	9/1997	Hintsche et al.	435/14
5,680,858	10/1997	Hansen et al.	435/14
5,682,233	10/1997	Brinda	435/14
5,695,623	12/1997	Michel et al.	435/14
5,708,247	1/1998	McAleer et al.	435/14
5,711,861	1/1998	Ward et al.	435/14
5,711,862	1/1998	Sakoda et al.	435/14
5,741,211	4/1998	Renirie et al.	435/14
5,791,344	8/1998	Schulman et al.	435/14

FOREIGN PATENT DOCUMENTS

0 026 995 A1	4/1981	(EP) .
0 048 090 A2	3/1982	(EP) .
0 078 636 A1	5/1983	(EP) .
0 096 288 A1	12/1983	(EP) .
0 125 139 A2	11/1984	(EP) .

0 127 958 A2	12/1984	(EP) .
0 136 362 A1	4/1985	(EP) .
0 170 375 A2	2/1986	(EP) .
0 177 743 A2	4/1986	(EP) .
0 080 304 B1	5/1986	(EP) .
0 184 909 A2	6/1986	(EP) .
0 206 218 A2	12/1986	(EP) .
0 230 472 A1	8/1987	(EP) .
0 241 309 A3	10/1987	(EP) .
0 245 073 A2	11/1987	(EP) .
0 278 647 A2	8/1988	(EP) .
0 359 831 A1	3/1990	(EP) .
0 368 209 A1	5/1990	(EP) .
0 390 390 A1	10/1990	(EP) .
0 400 918 A1	12/1990	(EP) .
0 453 283 A1	10/1991	(EP) .
0 470 290 A1	2/1992	(EP) .
0 127 958 B2	3/1992	(EP) .
0 255 291 B1	6/1992	(EP) .
1394171	5/1975	(GB) .
1599241	9/1981	(GB) .
2 073 891	10/1981	(GB) .
2 154 003	2/1988	(GB) .
2 204 408	11/1988	(GB) .
2 254 436	10/1992	(GB) .
54-41191	4/1979	(JP) .
55-10581	1/1980	(JP) .
55-10583	1/1980	(JP) .
55-10584	1/1980	(JP) .
55-12406	1/1980	(JP) .
56-163447	12/1981	(JP) .
57-70448	4/1982	(JP) .
60-173457	9/1985	(JP) .
60-173458	9/1985	(JP) .
60-173459	9/1985	(JP) .
61-90050	5/1986	(JP) .
62-85855	4/1987	(JP) .
62-114747	5/1987	(JP) .
63-58149	3/1988	(JP) .
63-128252	5/1988	(JP) .
63-139246	6/1988	(JP) .
63-294799	12/1988	(JP) .
63-317757	12/1988	(JP) .
63-317758	12/1988	(JP) .
1-114746	5/1989	(JP) .
1-114747	5/1989	(JP) .
1-124060	5/1989	(JP) .
1-134244	5/1989	(JP) .
1-156658	6/1989	(JP) .
2-62958	3/1990	(JP) .
2-120655	5/1990	(JP) .
2-287145	11/1990	(JP) .
2-310457	12/1990	(JP) .
3-26956	2/1991	(JP) .
3-28752	2/1991	(JP) .
3-202764	9/1991	(JP) .
5-72171	3/1993	(JP) .
5-196595	8/1993	(JP) .
7-72585	3/1995	(JP) .
1281988 A1	1/1987	(SU) .
WO 85/05119	11/1985	(WO) .
89/08713	9/1989	(WO) .
90/05300	5/1990	(WO) .
90/05910	5/1990	(WO) .
91/01680	2/1991	(WO) .
91/04704	4/1991	(WO) .
91/15993	10/1991	(WO) .
92/13271	8/1992	(WO) .
94/20602	9/1994	(WO) .
94/27140	11/1994	(WO) .
96/30431	10/1996	(WO) .
WO 96/30431	10/1996	(WO) .

US 6,284,478 B1

Page 4

97/02847	1/1997	(WO) .
97/19344	5/1997	(WO) .
97/42882	11/1997	(WO) .
97/42883	11/1997	(WO) .
97/42886	11/1997	(WO) .
97/42888	11/1997	(WO) .
97/43962	11/1997	(WO) .

OTHER PUBLICATIONS

Albery, W. J. et al., "Amperometric Enzyme Electrodes," *Phil. Trans. R. Soc. Lond.* B316:107–119 (1987).

Alcock, S. J. et al., "Continuous Analyte Monitoring to Aid Clinical Practice," *IEEE Engineering in Medicine and Biology*, 319–325 (1994).

Anderson, L. B. et al., "Thin–Layer Electrochemistry: Steady–State Methods of Studying Rate Processes," *J. Electroanal. Chem.*, 10:295–395 (1965).

Bartlett, P. N. et al., "Covalent Binding of Electron Relays to Glucose Oxidation," *J. Chem. Soc. Chem. Commun.*, 1603–1604 (1987).

Bartlett, P. N. et al., "Modification of glucose oxidase by tetrathiafulvalene," *J. Chem. Soc., Chem. Commun.*, 16 (1 page–Abstract only) (1990).

Bartlett, P. N. et al., "Strategies for the Development of Amperometric Enzyme Electrodes," *Biosensors*, 3:359–379 (1987/88).

Bindra, D.S. et al., "Design and in Vitro Studies of a Needle–Type Glucose Sensor for Subcutaneous Monitoring", *Anal. Chem.*, 63(17):1692–1696 (Sep. 1, 1991).

Bobbioni–Harsch, E. et al., "Lifespan of subcutaneous glucose sensors and their performances during dynamic glycaemia changes in rats," *J. Biomed. Eng.* 15:457–463 (1993).

Brandt, J. et al., "Covalent attachment of proteins to polysaccharide carriers by means of benzoquinone," *Biochim. Biophys. Acta*, 386(1)(1 page Abstract only) (1975).

Brownlee, M. et al., "A Glucose–Controlled Insulin–Delivery System: Semisynthetic Insulin Bound to Lectin", *Science*, 206(4423):1190–1191 (Dec. 7, 1979).

Cass, A.E.G. et al., "Ferricinum Ion As An Electron Acceptor for Oxido–Reductases," *J. Electroanal. Chem.*, 190:117–127 (1985).

Cass, A.E.G. et al., "Ferrocene–Mediated Enzyme Electrode for Amperometric Determination of Glucose", *Anal. Chem.*, 56(4):667–671 (Apr. 1984).

Castner, J. F. et al., "Mass Transport and Reaction Kinetic Parameters Determined Electrochemically for Immobilized Glucose Oxidase," *Biochemisty*, 23(10):2203–2210 (1984).

Claremont, D.J. et al., "Biosensors for Continuous In Vivo Glucose Monitoring", *IEEE Engineering in Medicine and Biology Society 10th Annual International Conference*, New Orleans, Louisiana, 3 pgs. (Nov. 4–7, 1988).

Clark, L.C. et al., "Differential Anodic Enzyme Polarography for the Measurement of Glucose", *Oxygen Transport to Tissue: Instrumentation, Methods, and Physiology*, 127–132 (1973).

Clark, L.C., Jr. et al., "Electrode Systems for Continuous Monitoring in Cardiovascular Surgery," *Annals New York Academy of Sciences*, pp. 29–45 (1962).

Clark, L.C. et al., "Long–term Stability of Electroenzymatic Glucose Sensors Implanted in Mice," *Trans. Am. Soc. Artif. Intern. Organs*, XXXIV:259–265 (1988).

Clarke, W. L., et al., "Evaluating Clinical Accuracy of Systems for Self–Monitoring of Blood Glucose," *Diabetes Care*, 10(5):622–628 (Sep.–Oct. 1987).

Csöregi, E. et al., "Design, Characterization, and One–Point in Vivo Calibration of a Subcutaneously Implanted Glucose Electrode," *Anal. Chem.* 66(19):3131–3138 (Oct. 1, 1994).

Csöregi, E. et al., "Design and Optimization of a Selective Subcutaneously Implantable Glucose Electrode Based on "Wired" Glucose Oxidase," *Anal. Chem.* 67(7):1240–1244 (Apr. 1, 1995).

Csöregi, E. et al., "On–Line Glucose Monitoring by Using Microdialysis Sampling and Amperometric Detection Based on "Wired" Glucose Oxidase in Carbon Paste," *Mikrochim. Acta.* 121:31–40 (1995).

Davis, G., "Electrochemical Techniques for the Development of Amperometric Biosensors", *Biosensors*, 1:161–178 (1985).

Degani, Y. et al., "Direct Electrical Communication between Chemically Modified Enzymes and Metal Electrodes. 1. Electron Transfer from Glucose Oxidase to Metal Electrodes via Electron Relays, Bound Covalently to the Enzyme," *J. Phys. Chem.*, 91(6):1285–1289 (1987).

Degani, Y. et al., "Direct Electrical Communication between Chemically Modified Enzymes and Metal Electrodes. 2. Methods for Bonding Electron–Transfer Relays to Glucose Oxidase and D–Amino–Acid Oxidase," *J. Am. Chem. Soc.*, 110(8):2615–2620 (1988).

Degani, Y. et al., "Electrical Communication between Redox Centers of Glucose Oxidase and Electrodes via Electrostatically and Covalently Bound Redox Polymers," *J. Am. Chem. Soc.*, 111:2357–2358 (1989).

Denisevich, P. et al., "Unidirectional Current Flow and Charge State Trapping at Redox Polymer Interfaces on Bilayer Electrodes: Principles, Experimental Demonstration, and Theory," *J. Am. Chem. Soc.*, 103(16):4727–4737 (1981).

Dicks, J. M., "Ferrocene modified polypyrrole with immobilised glucose oxidase and its application in amperometric glucose microbiosensors," *Ann. Biol. clin.*, 47:607–619 (1989).

Engstrom, R.C., "Electrochemical Pretreatment of Glassy Carbon Electrodes", *Anal. Chem.*, 54(13):2310–2314 (Nov. 1982).

Engstrom, R.C. et al., "Characterization of Electrochemically Pretreated Glassy Carbon Electrodes", *Anal. Chem.*, 56(2):136–141 (Feb. 1984).

Ellis, C. D., "Selectivity and Directed Charge Transfer through an Electroactive Metallopolymer Film," *J. Am. Chem. Soc.*, 103(25):7480–7483 (1981).

Feldman, B.J. et al., "Electron Transfer Kinetics at Redox Polymer/Solution Interfaces Using Microelectrodes and Twin Electrode Thin Layer Cells", *J. Electroanal. Chem.*, 194(1):63–81 (Oct. 10, 1985).

Fischer, H. et al., "Intramolecular Electron Transfer Mediated by 4,4'–Bipyridine and Related Bridging Groups", *J. Am. Chem. Soc.*, 98(18):5512–5517 (Sep. 1, 1976).

Foulds, N.C. et al., "Enzyme Entrapment in Electrically Conducting Polymers," *J. Chem. Soc., Faraday Trans 1.*, 82:1259–1264 (1986).

Foulds, N.C. et al., "Immobilization of Glucose Oxidase in Ferrocene–Modified Pyrrole Polymers," *Anal. Chem.*, 60(22):2473–2478 (Nov. 15, 1988).

Frew, J.E. et al., "Electron–Transfer Biosensors", *Phil. Trans. R. Soc. Lond.*, B316:95–106 (1987).

Gorton, L. et al., "Selective detection in flow analysis based on the combination of immobilized enzymes and chemically modified electrodes," *Analytica Chimica Acta.*, 250:203–248 (1991).

US 6,284,478 B1

Page 5

Gregg, B. A. et al., "Cross–Linked Redox Gels Containing Glucose Oxidase for Amperometric Biosensor Applications," *Analytical Chemistry*, 62(3):258–263 (Feb. 1, 1990).

Gregg, B. A. et al., "Redox Polymer Films Containing Enzymes. 1. A Redox–Conducting Epoxy Cement: Synthesis, Characterization, and Electrocatalytic Oxidation of Hydroquinone," *J. Phys. Chem.*, 95(15):5970–5975 (1991).

Hale, P.D. et al., "A New Class of Amperometric Biosensor Incorporating a Polymeric Electron–Transfer Mediator," *J. Am. Chem. Soc.*, 111(9):3482–3484 (1989).

Harrison, D.J. et al., "Characterization of Perfluorosulfonic Acid Polymer Coated Enzyme Electrodes and a Miniaturized Integrated Potentiostat for Glucose Analysis in Whole Blood", *Anal. Chem.*, 60(19):2002–2007 (Oct. 1, 1988).

Hawkridge, F. M. et al., "Indirect Coulometric Titration of Biological Electron Transport Components," *Analytical Chemistry*, 45(7):1021–1027 (Jun. 1973).

Heller, A., "Amperometric biosensors based on three–dimensional hydrogel–forming epoxy networks," *Sensors and Actuators B*, 13–14:180–183 (1993).

Heller, A., "Electrical Connection of Enzyme Redox Centers to Electrodes," *J. Phys. Chem.*, 96(9):3579–3587 (1992).

Heller, A., "Electrical Wiring of Redox Enzymes," *Acc. Chem. Res.*, 23(5):129–134 (1990).

Ianniello, R.M. et al. "Immobilized Enzyme Chemically Modified Electrode as an Amperometric Sensor", *Anal. Chem.*, 53(13):2090–2095 (Nov. 1981).

Ianniello, R.M. et al., "Differential Pulse Voltammetric Study of Direct Electron Transfer in Glucose Oxidase Chemically Modified Graphite Electrodes", *Anal. Chem.*, 54:(7):1098–1101 (Jun. 1981).

Ikeda, T. et al., "Glucose oxidase–immobilized benzoquinone–carbon paste electrode as a glucose sensor," *Agric. Biol. Chem.*, 49(2) (1 page–Abstract only)(1985).

Ikeda, T. et al., "Kinetics of Outer–Sphere Electron Transfers Between Metal Complexes in Solutions and Polymeric Films on Modified Electrodes", *J. Am. Chem. Soc.*, 103(25):7422–7425 (Dec. 16, 1981).

Johnson, J. M. et al., "Potential–Dependent Enzymatic Activity in an Enzyme Thin–Layer Cell," *Anal. Chem.* 54:1377–1383 (1982).

Johnson, K.W., "Reproducible Electrodeposition of Biomolecules for the Fabrication of Miniature Electroenzymatic Biosensors," *Sensors and Actuators B Chemical*, B5:85–89 (1991).

Jönsson, G. et al., "An Amperometric Glucose Sensor Made by Modification of a Graphite Electrode Surface With Immobilized Glucose Oxidase and Adsorbed Mediator", *Biosensors*, 1:355–368 (1985).

Josowicz, M. et al., "Electrochemical Pretreatment of Thin Film Platinum Electrodes", *J. Elecrochem. Soc.*, 135(1):112–115 (Jan. 1988).

Katakis, I. et al., "Electrostatic Control of the Electron Transfer Enabling Binding of Recombinant Glucose Oxidase and Redox Polyelectrolytes," *J. Am. Chem. Soc.*, 116(8):3617–3618 (1994).

Katakis, I. et al., "L–α–Glycerophosphate and L–Lactate Electrodes Based on the Electrochemical "Wiring" of Oxidases," *Analytical Chemisry*, 64(9):1008–1013 (May 1, 1992).

Kenausis, G. et al., "'Wiring' of glucose oxidase and lactate oxidase within a hydrogel made with poly(vinyl pyridine) complexed with [Os(4,4'–dimethoxy–2,2'–bipyridine)$_2$ Cl]$^{+/2+}$," *J. Chem. Soc., Faraday Trans.*, 92(20):4131–4136 (1996).

Koudelka, M. et al., "In–Vivo Behaviour of Hypodermically Implanted Microfabricated Glucose Sensors", *Biosensors & Bioelectronics*, 6(1):31–36 (1991).

Kulys, J. et al., "Mediatorless peroxidase electrode and preparation of bienzyme sensors," *Bioelectrochemisty and Bioenergetics*, 24:305–311 (1990).

Lager, W. et al., "Implantable Electrocatalytic Glucose Sensor," *Horm. Metab. Res.*, 26:526–530 (Nov. 1994).

Lindner, E. et al. "Flexible (Kapton–Based) Microsensor Arrays of High Stability for Cardiovascular Applications",*J. Chem. Soc.Faraday Trans.*, 89(2):361–367 (Jan. 21, 1993).

Maidan, R. et al., "Elimination of Electrooxidizable Interferant–Produced Currents in Amperometric Biosensors," *Analytical Chemistry*, 64(23):2889–2896 (Dec. 1, 1992).

Mastrototaro, J.J. et al., "An Electroenzymatic Glucose Sensor Fabricated on a Flexible Substrate", *Sensors and Biosensors B Chemical*, B5:139–144 (1991).

McNeil, C. J. et al., "Thermostable Reduced Nicotinamide Adenine Dinucleotide Oxidase: Application to Amperometric Enzyme Assay," *Anal. Chem.*, 61(1):25–29 (Jan. 1, 1989).

Miyawaki, O. et al., "Electrochemical and Glucose Oxidase Coenzyme Activity of Flavin Adenine Dinucleotide Covalently Attached to Glassy Carbon at the Adenine Amino Group", *Biochimica et Biophysica Acta*, 838:60–68 (1985).

Moatti–Sirat, D. et al., "Evaluating in vitro and in vivo the inteference of ascorbate and acetaminophen on glucose detection by a needle–type glucose sensor," *Biosensors & Bioelectronics*, 7(5):345–352 (1992).

Moatti–Sirat, D. et al., "Reduction of acetaminophen interference in glucose sensors by a composite Nafion membrane: demonstration in rats and man," *Diabetologia*, 37(6) (1 page–Abstract only) (Jun. 1994).

Moatti–Sirat, D. et al., "Towards continuous glucose monitoring: in vivo evaluation of a miniaturized glucose sensor implanted for several days in rat subcutaneous tissue," *Diabetologia*, 35(3) (1 page–Abstract only) (Mar. 1992).

Nagy, G. et al., "A New Type of Enzyme Electrode: The Ascorbic Acid Eliminator Electrode," *Life Sciences*, 31(23):2611–2616 (1982).

Nakamura, S. et al., "Effect of Periodate Oxidation on the Structure and Properties of Glucose Oxidase," *Biochimica et Biophysica Acta.*, 445:294–308 (1976).

Narazimhan, K. et al., "p–Benzoquinone activation of metal oxide electrodes for attachment of enzymes," *Enzyme Microb. Technol.*, 7(6)(1 page–Abstract only)(1985).

Ohara, T. J. et al., "Glucose Electrodes Based on Cross–Linked [Os(bpy)$_2$Cl]$^{+/2+}$ Complexed Poly(1–vinylimadazole) Films," *Analytical Chemistry*, 65(23):3512–3516 (Dec. 1, 1993).

Ohara, T. J., "Osmium Bipyridyl Redox Polymers Used in Enzyme Electrodes," *Platinum Metals Rev.*, 39(2):54–62 (Apr. 1995).

Ohara, T. J. et al., ""Wired" Enzyme Electrodes for Amperometric Determination of Glucose or Lactate in the Presence of Interfering Substances," *Analytical Chemistry*, 66(15):2451–2457 (Aug. 1, 1994).

Olievier, C. N. et al., "In vivo Measurement of Carbon Dioxide Tension with a Miniature Electrode," *Pflugers Arch.* 373:269–272 (1978).

Paddock, R. et al., "Electrocatalytic reduction of hydrogen peroxide via direct electron transfer from pyrolytic graphite electrodes to irreversibly adsorbed cytochrome c peroxidase," *J. Electroanal. Chem.*, 260:487–494 (1989).

US 6,284,478 B1

Page 6

Palleschi, G. et al., "A Study of Interferences in Glucose Measurements in Blood by Hydrogen Peroxide Based Glucose Probes", *Anal. Biochem.*, 159:114–121 (1986).

Pankratov, I. et al., "Sol–gel derived renewable–surface biosensors," *Journal of Electroanalytical Chemistry*, 393:35–41 (1995).

Pathak, C. P. et al., "Rapid Photopolymerization of Immunoprotective Gels in Contact with Cells and Tissue," *J. Am. Chem. Soc.*, 114(21):8311–8312 (1992).

Pickup, J., "Developing glucose sensors for in vivo use," *Tibtech*, 11: 285–289 (Jul. 1993).

Pickup, J. C. et al., "In vivo molecular sensing in diabetes mellitus: an implantable glucose sensor with direct electron transfer," *Diabetologia*, 32(3):213–217 (1989).

Pickup, J. et al., "Potentially–implantable, amperometric glucose sensors with mediated electron transfer: improving the operating stability," *Biosensors*, 4(2) (1 page–Abstract only) (1989).

Pishko, M.V. et al., "Amperometric Glucose Microelectrodes Prepared Through Immobilization of Glucose Oxidase in Redox Hydrogels", *Anal. Chem.*, 63(20):2268–2272 (Oct. 15, 1991).

Poitout, V. et al., "A glucose monitoring system for on line estimation in man of blood glucose concentration using a miniaturized glucose sensor implanted in the subcutaneous tissue and a wearable control unit," *Diabetolgia*, 36(7) (1 page–Abstract only) (Jul. 1993).

Poitout, V. et al., "Calibration in dogs of a subcutaneous miniaturized glucose sensor using a glucose meter for blood glucose determination," *Biosensors & Bioelectronics*, 7:587–592 (1992).

Poitout, V. et al., "In vitro and in vivo evaluation in dogs of a miniaturized glucose sensor," *ASAIO Transactions*, 37(3) (1 page–Abstract only) (Jul.–Sep. 1991).

Pollak, A. et al., "Enzyme Immobilization by Condensation Copolymerization into Cross–Linked Polyacrylamide Gels," *J. Am. Chem. Soc.*, 102(20):6324–6336 (1980).

Reach, G. et al., "Can Continuous Glucose Monitoring Be Used for the Treatment of Diabetes?" *Analytical Chemistry*, 64(6):381–386 (Mar. 15, 1992).

Rebrin, K. et al., "Automated Feedback Control of Subcutaneous Glucose Concentration in Diabetic Dogs", *Diabetologia*, 32(8):573–576 (Aug. 1989).

Sakakida, M. et al., "Ferrocene–mediate needle–type glucose sensor covered with newly designed biocompatible membrane," *Sensors and Actuators B*, 13–14:319–322 (1993).

Samuels, G. J. et al., "An Electrode–Supported Oxidation Catalyst Based on Ruthenium (IV). pH "Encapsulation" in a Polymer Film," *J. Am. Chem. Soc.*, 103(2):307–312 (1981).

Sasso, S.V. et al., "Electropolymerized 1,2–Diaminobenzene as a Means to Prevent Interferences and Fouling and to Stabilize Immobilized Enzyme in Electrochemical Biosensors", *Anal. Chem.*, 62(11):1111–1117 (Jun. 1, 1990).

Scheller, F. et al., "Enzyme electrodes and their application," *Phil. Trans. R. Soc, Lond.*, B 316:85–94 (1987).

Schmehl, R.H. et al., "The Effect of Redox Site Concentration on the Rate of Mediated Oxidation of Solution Substrates by a Redox Copolymer Film", *J. Electroanal. Chem.*, 152:97–109 (Aug. 25, 1983).

Shichiri, M. et al., "Glycaemic Control in Pancreatetomized Dogs with a Wearable Artificial Endocrine Pancreas", *Diabetologia*, 24(3):179–184 (Mar. 1983).

Sittampalam, G. et al., "Surface–Modified Electrochemical Detector for Liquid Chromatography", *Anal. Chem.*, 55(9):1608–1610 (Aug. 1983).

Soegijoko, S. et al., *Horm. Metabl. Res., Suppl. Ser*, 12 (1 page–Abstract only) (1982).

Sprules, S. D. et al., "Evaluation of a New Disposable Screen–Printed Sensor Strip for the Measurement of NADH and Its Modification to Produce a Lactate Biosensor Employing Microliter Volumes," *Electroanalysis*, 8(6):539–543 (1996).

Sternberg, F. et al., "Calibration Problems of Subcutaneous Glucosensors when Applied "In–Situ" in Man," *Horm. metabl. Res*, 26:524–525 (1994).

Sternberg, R. et al., "Covalent Enzyme Coupling on Cellulose Acetate Membranes for Glucose Sensor Development," *Analytical Chemistry*, 60(24):2781–2786 (Dec. 15, 1988).

Sternberg, R. et al., "Study and Development of Multilayer Needle–type Enzyme–based Glucose Microsensors," *Biosensors*, 4:27–40 (1988).

Suekane, M., "Immobolization of glucose isomerase," *Zeitschrift für Allgemeine Mikrobiologie*, 22(8):565–576 (1982).

Tajima, S. et al., "Simultaneous Determination of Glucose and 1,5–Anydroglucitol", *Chemical Abstracts*, 111(25):394 111:228556g (Dec. 18, 1989).

Tarasevich, M.R. "Bioelectrocatalysis", *Comprehensive Treatise of Electrochemistry*, 10(Ch. 4):231–295 (1985).

Tatsuma, T. et al., "Enzyme Monolayer– and Bilayer–Modified Tin Oxide Electrodes for the Determination of Hydrogen Peroxide and Glucose," *Anal. Chem.*, 61(21):2352–2355 (Nov. 1, 1989).

Taylor, C. et al., "'Wiring' of glucose oxidase within a hydrogel made with polyvinyl imidazole complexed with [(Os–4,4'–dimethoxy–2,2'–bipyridine)Cl]$^{+/2+}$," *Journal of Electroanalytical Chemistry*, 396:511–515 (1995).

Trojanowicz, M. et al., "Enzyme Entrapped Polypyrrole Modified Electrode for Flow–Injection Determination of Glucose," *Biosensors & Bioelectronics*, 5:149–156 (1990).

Turner, A.P.F. et al., "Diabetes Mellitus: Biosensors for Research and Management", *Biosensors*, 1:85–115 (1985).

Turner, R.F.B. et al., "A Biocompatible Enzyme Electrode for Continuous in vivo Glucose Monitoring in Whole Blood," *Sensors and Actuators*, B1(1–6):561–564 (Jan. 1990).

Tuzhi, P. et al., "Constant Potential Pretreatment of Carbon Fiber Electrodes for In Vivo Electrochemistry", *Analytical Letters*, 24(6):935–945 (1991).

Umaha, M., "Protein–Modified Electrochemically Active Biomaterial Surface," *U.S. Army Research Office Report*, (12 pages) (Dec. 1988).

Urban, G. et al., "Miniaturized Thin–Film Biosensors Using Covalently Immobilized Glucose Oxidase", *Biosensors & Bioelectronics*, 6(7):555–562 (1991).

Velho, G. et al., "In Vitro and In Vivo Stability of Electrode Potentials in Needle–Type Glucose Sensors", *Diabetes*, 38(2):164–171 (Feb. 1989).

Velho, G. et al., "Strategies for calibrating a subcutaneous glucose sensor," *Biomed. Biochin. Acta*, 48(11/12):957–964 (1989).

Von Woedtke, T. et al., "In Situ Calibration of Implanted Electrochemical Glucose Sensors," *Biomed. Biochim. Acta*, 48(11/12):943–952 (1989).

US 6,284,478 B1

Page 7

Vreeke, M.S. et al., "Chapter 15: Hydrogen Peroxide Electrodes Based on Electrical Connection of Redox Centers of Various Peroxidases to Electrodes through a Three–Dimensional Electron–Relaying Polymer Network," *Diagnostic Biosensor Polymers*, 7 pgs. (Jul. 26, 1993).

Vreeke, M. et al., "Hydrogen Peroxide and β–Nicotinamide Adenine Dinucleotide Sensing Amperometric Electrodes Based on Electrical Connection of Horseradish Peroxidase Redox Centers to Electrodes through a Three–Dimensional Electron Relaying Polymer Network," *Analytical Chemistry*, 64(24):3084–3090 (Dec. 15, 1992).

Wang, J. et al., "Activation of Glassy Carbon Electrodes by Alternating Current Electrochemical Treatment", *Analytica Chimica Acta*, 167:325–334 (Jan. 1985).

Wang, J. et al., "Amperometric biosensing of organic peroxides with peroxidase–modified electrodes," *Analytica Chimica Acta*. 254:81–88 (1991).

Wang, D. L. et al., "Miniaturized Flexible Amperometric Lactate Probe," *Analytical Chemistry*, 65(8):1069–1073 (Apr. 15, 1993).

Wang, J. et al., "Screen–Printable Sol–Gel Enzyme–Containing Carbon Inks," *Analytical Chemistry*, 68(15):2705–2708 (Aug. 1, 1996).

Wang, J. et al., "Sol–Gel–Derived Metal–Dispersed Carbon Composite Amperometric Biosensors," *Electroanalysis*, 9(1):52–55 (1997).

Williams, D.L. et al., "Electrochemical–Enzymatic Analysis of Blood Glucose and Lactate", *Anal. Chem.*, 42(1):118–121 (Jan. 1970).

Wilson, G. S. et al., "Progress toward the Development of an Implantable Sensor for Glucose," *Clinical Chemistry*, 38(9):1613–1617 (1992).

Yabuki, S. et al., "Electro–conductive Enzyme Membrane," *J. Chem. Soc. Chem. Commun*, 945–946 (1989).

Yang, L. et al., "Determination of Oxidase Enzyme Substrates Using Cross–Flow Thin–Layer Amperometry," *Electroanalysis*, 8(8–9):716–721 (1996).

Yao, S.J. et al., "The Interference of Ascorbate and Urea in Low–Potential Electrochemical Glucose Sensing", *Proceedings of the Twelfth Annual International Conference of the IEEE Engineering in Medicine and Biology Society*, 12(2):487–489 (Nov. 1–4, 1990).

Yao, T. et al., "A Chemically–Modified Enzyme Membrane Electrode As An Amperometric Glucose Sensor," *Analytica Chimica Acta.*, 148:27–33 (1983).

Ye, L. et al., "High Current Density "Wired" Quinoprotein Glucose Dehydrogenase Electrode," *Anal. Chem.*, 65(3):238–241 (Feb. 1, 1993).

Yildiz, A. et al., "Evaluation of an Improved Thin–Layer Electrode," *Analytical Chemistry*, 40(70):1018–1024 (Jun. 1968).

Zamzow, K. et al., New Wearable Continuous Blood Glucose Monitor (BGM) and Artificial Pancreas (AP), *Diabetes*, 39:5A(20) (May 1990).

Zhang, Y. et al., "Application of cell culture toxicity tests to the development of implantable biosensors," *Biosensors & Bioelectronics*, 6:653–661 (1991).

Zhang, Y. et al., "Elimination of the Acetaminophen Interference in an Implantable Glucose Sensor," *Anal. Chem.* 66:1183–1188 (1994).

Aisenberg et al., "Blood glucose, level monitoring alarm system," Great Britain Patent GB 1394171, issued May 14, 1975, (Abstract only).

Cerami, "Monitor for continuous in vivo measurement of glucose concentration," United States Patent 4,436,094, issued Mar. 13, 1984, 2 pages (Abstract only).

Franetzki, "Implantable, calibrateable measuring instrument for a body substance and a calibrating method," United States Patent 4,759,371, issued Jul. 26, 1988, 2 pages (Abstract only).

Gilli, "Apparatus and method employing plural electrode configurations for cardioversi on of atrial fibrillation in an arrhythmia control system," United States Patent 5,209,229, issued May 11, 1993, 2 pgs (Abstract only).

Klein, "Control and regulation device for glycemia," Great Britain Patent 1599241A, issued Sep. 30, 1981 (Abstract only).

Klein, "Method and apparatus for the control and regulation of glycemia," United States Patent 4,206,755, issued Jun. 10, 1980, 2 pages (Abstract only).

Lawton, "Implantable electrochemical sensor," United States Patent 4,016,866, issued Apr. 12, 1977, 2 pages (Abstract only).

Vadgama et al., "Sensor devices," United States Patent 5,531,878, issued Jul. 2, 1996, 2 pages (Abstract only).

Abstract from Korf, J. et al., "Monitoring of Glucose and Lactate Using Microdialysis: Applications in Neonates and Rat Brain", *Developmental Neuroscience*, vol. 15, No. 3–5, pp. 240–46 (1993).

Flentge F. et al., "An Enzyne–Reactor for Electrochemical Monitoring of Choline and Acetylcholine: Applications in High–Performance Liquid Chromatography, Brain Tissue, Microdialysis and Cerebrospinal Fluid", *Analytical Biochemistry*, vol. 204, No. 2, pp. 305–310, (Aug. 1, 1992).

Laurell, T., "A Continuous Glucose Monitoring System Based on Microdialysis", *Journal of Med. Eng. & Tech.*, vol. 16, No. 5, pp. 187–193 (Sep./Oct. 1992).

Marko–Varga, G. et al., "Enzyme–Based Biosensor as a Selective Detection Unit in Column Liquid Chromatography", *Journal of Chromatography A*, vol. 660, pp. 153–167 (1994).

Schmidt, F.J. et al., "Calibration of a Wearable Glucose Sensor", *The International Journal of Artificial Organs*, vol. 15, No. 1, pp. 55–61 (1992).
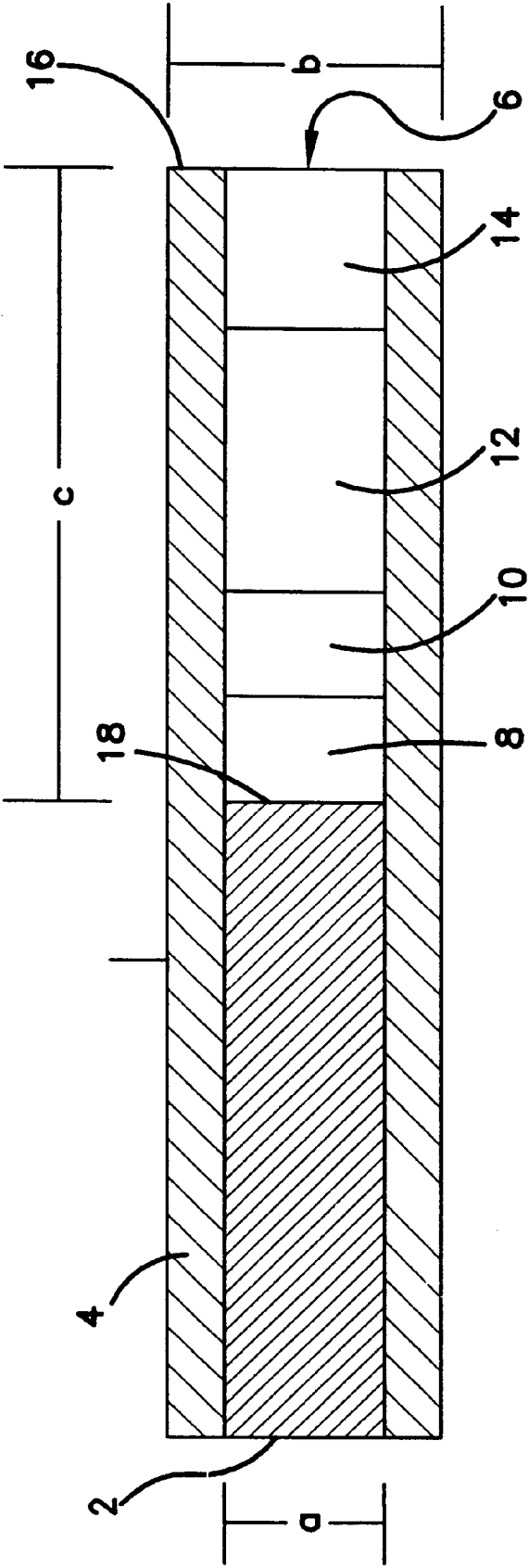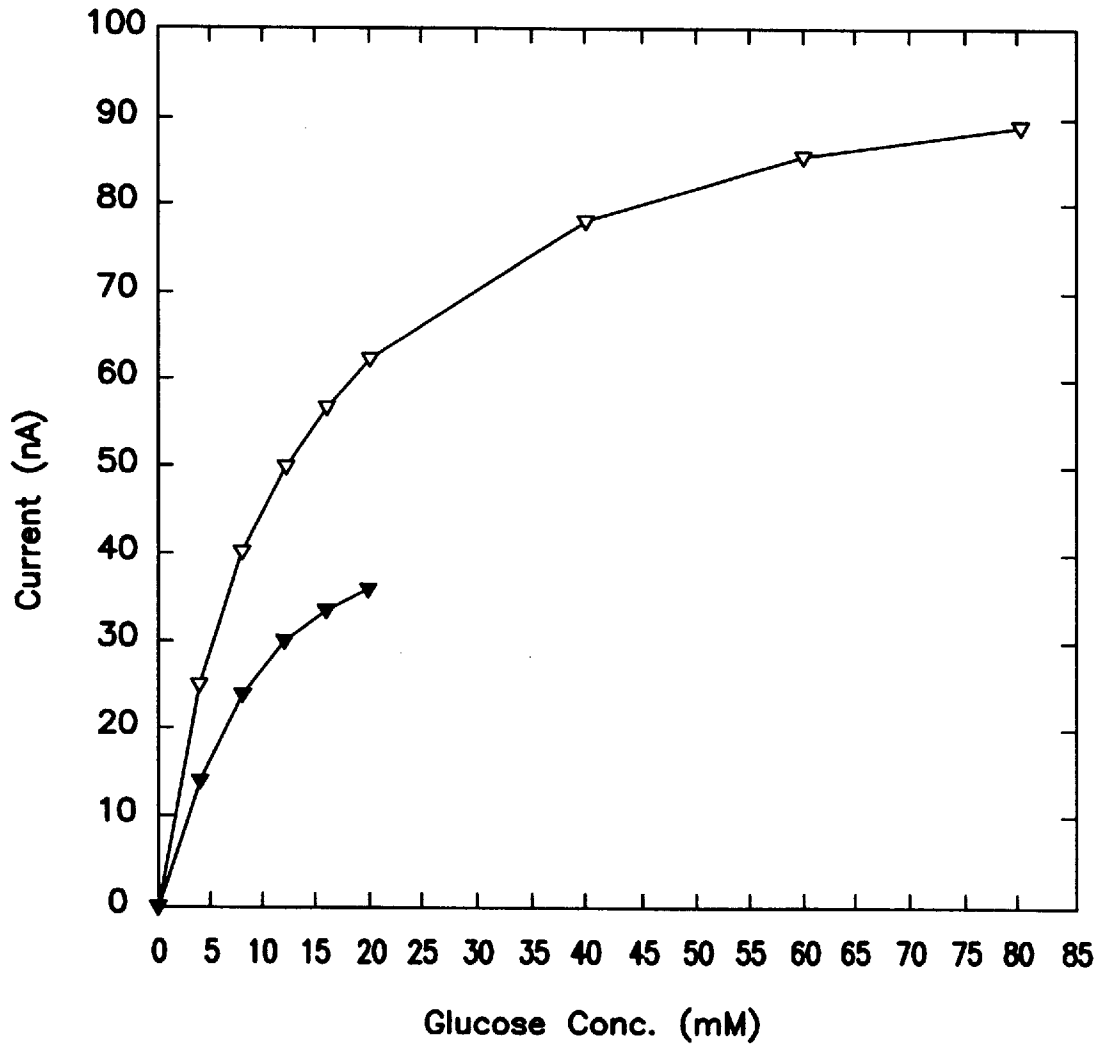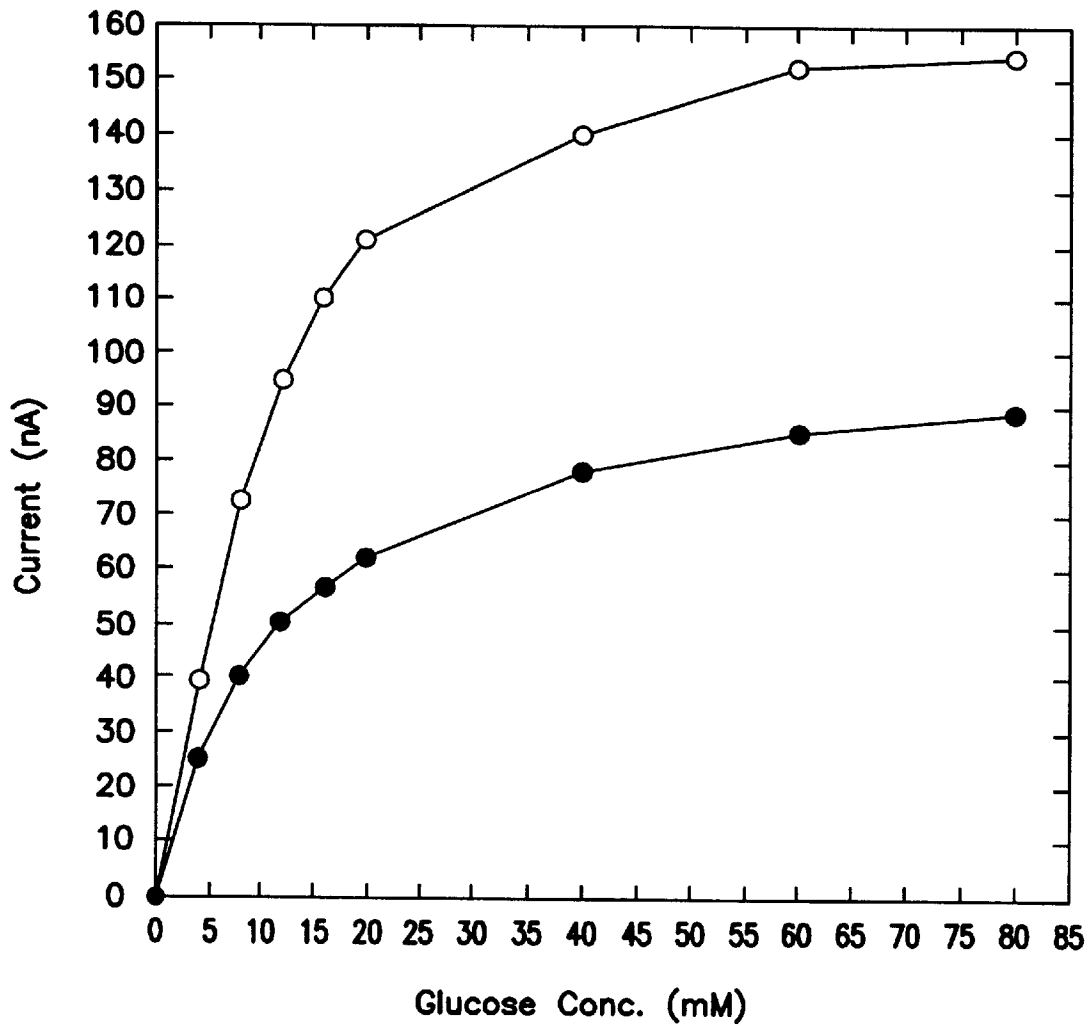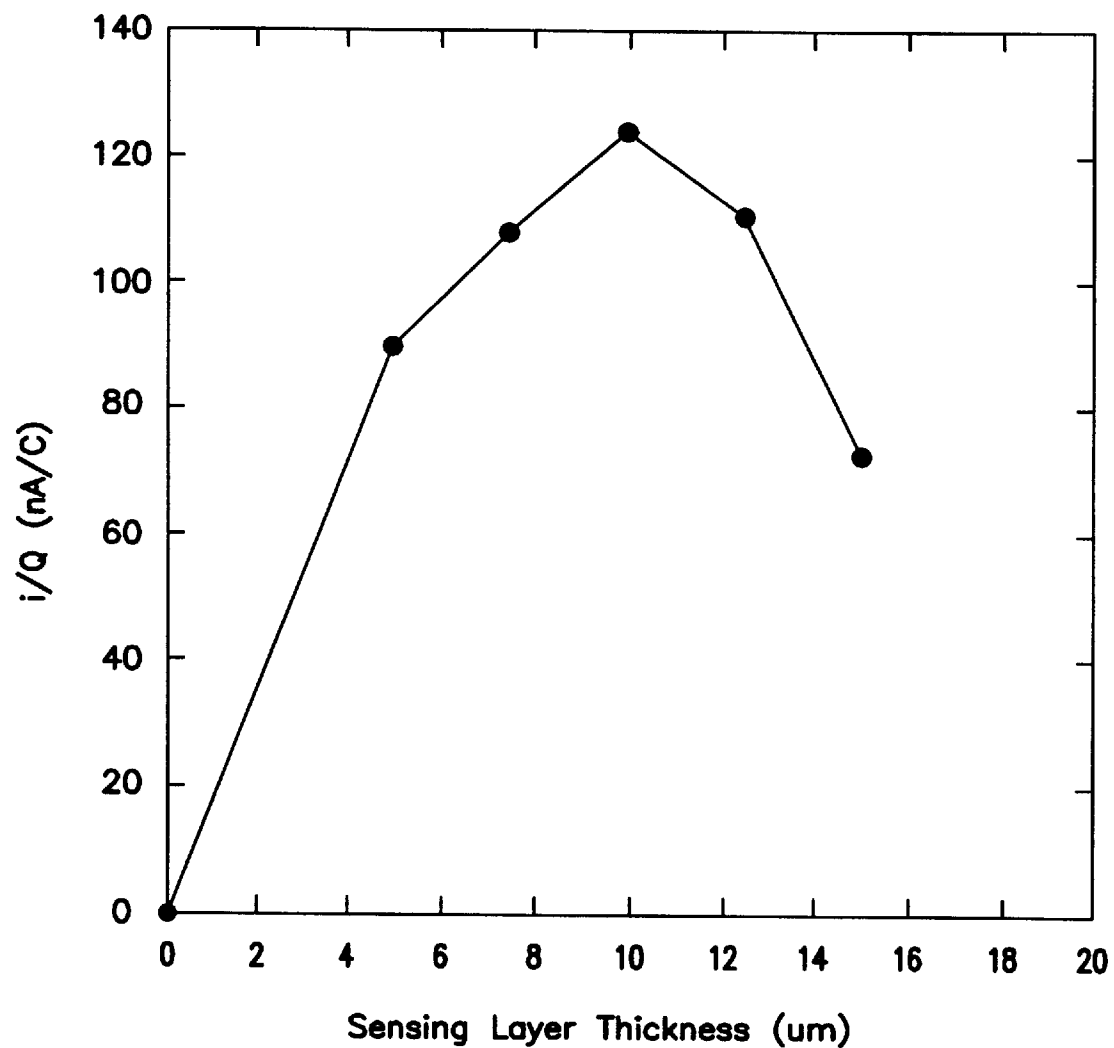
FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 6
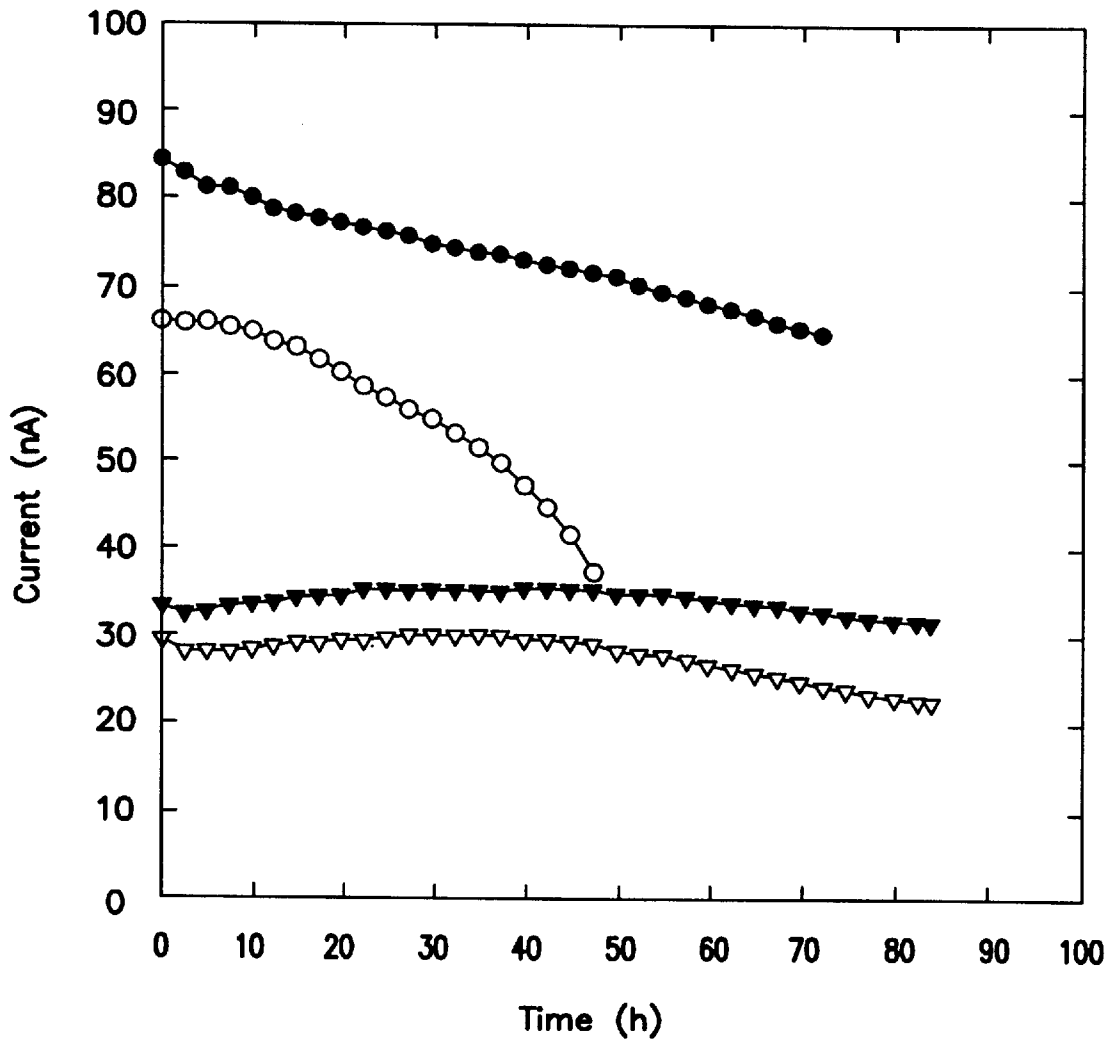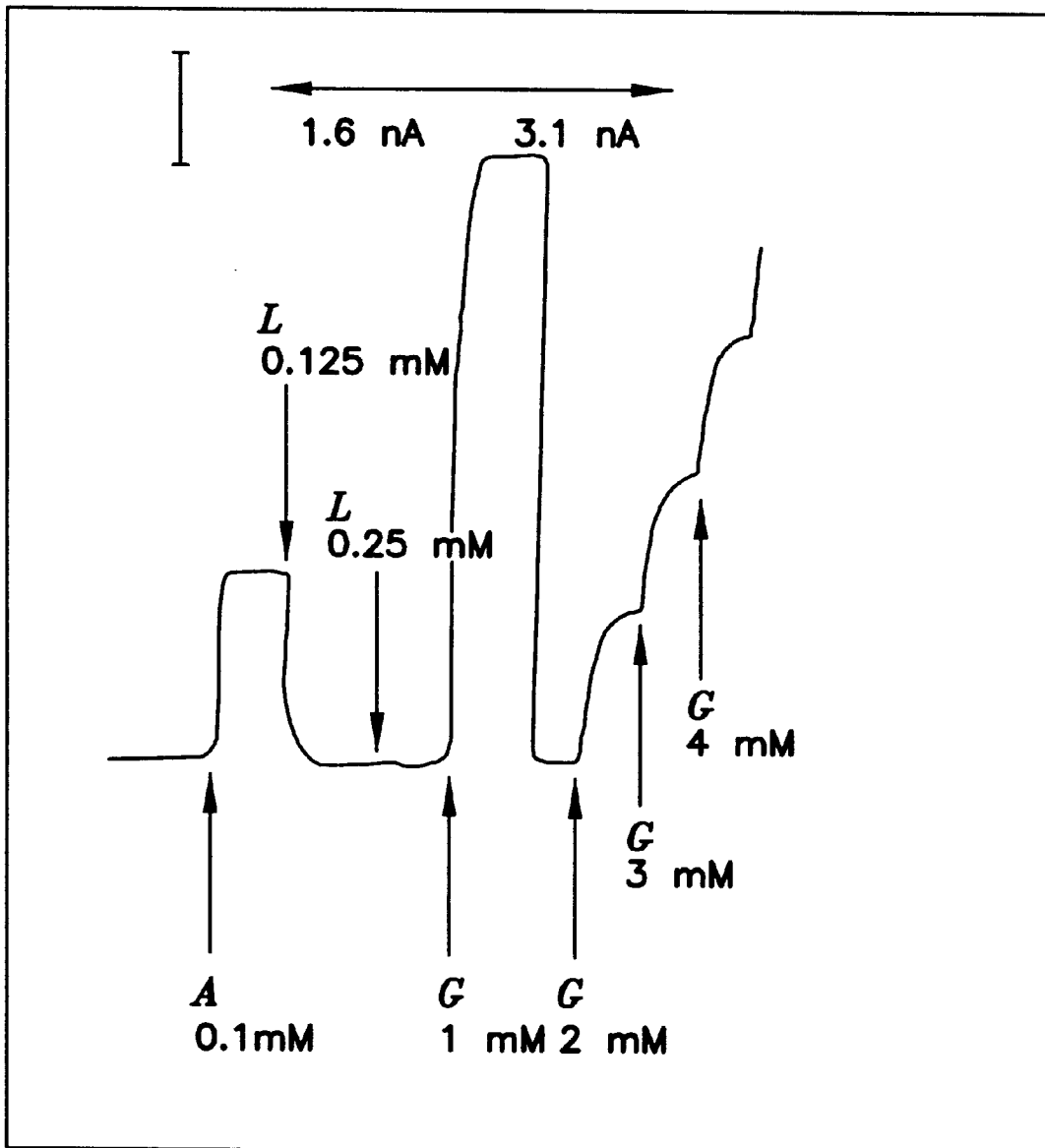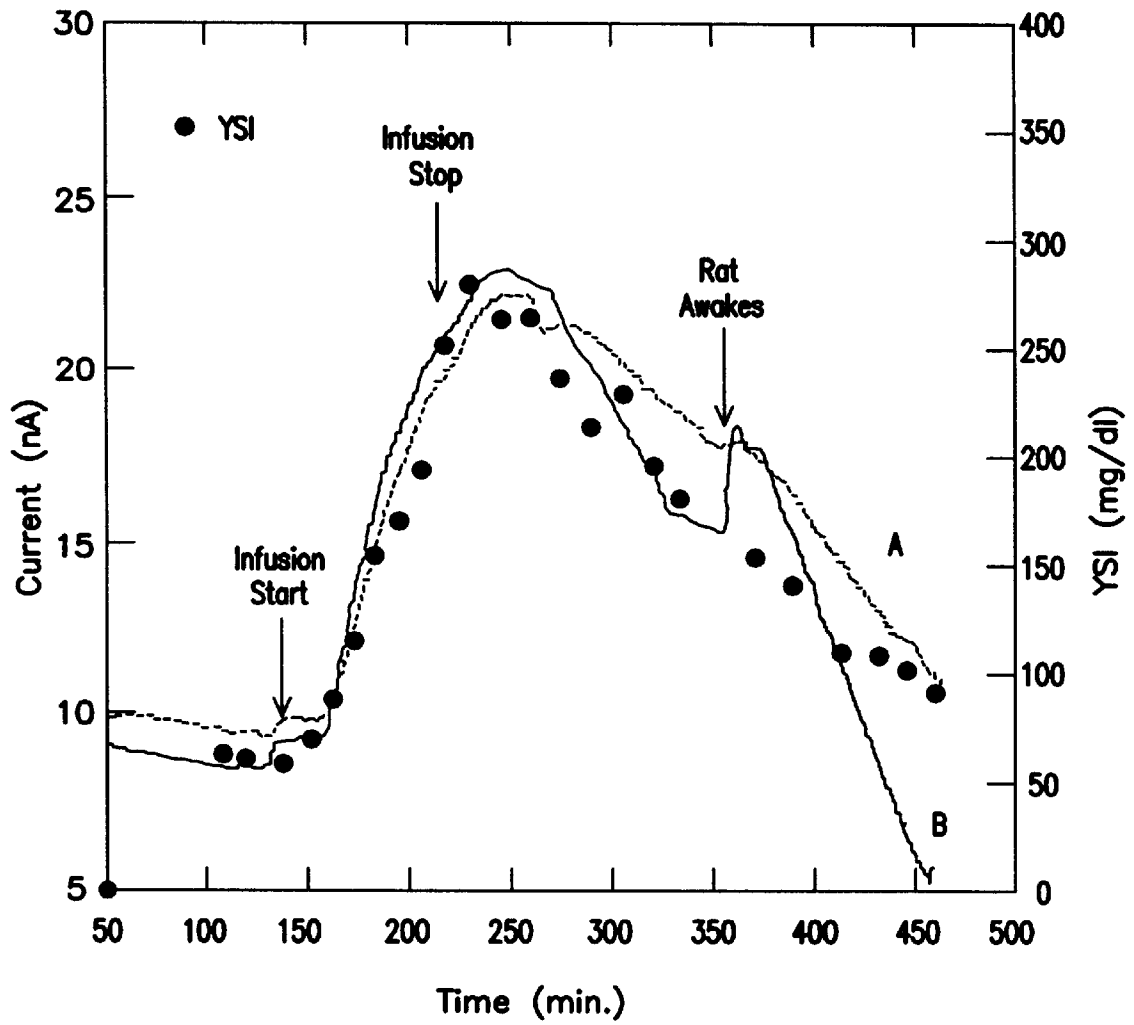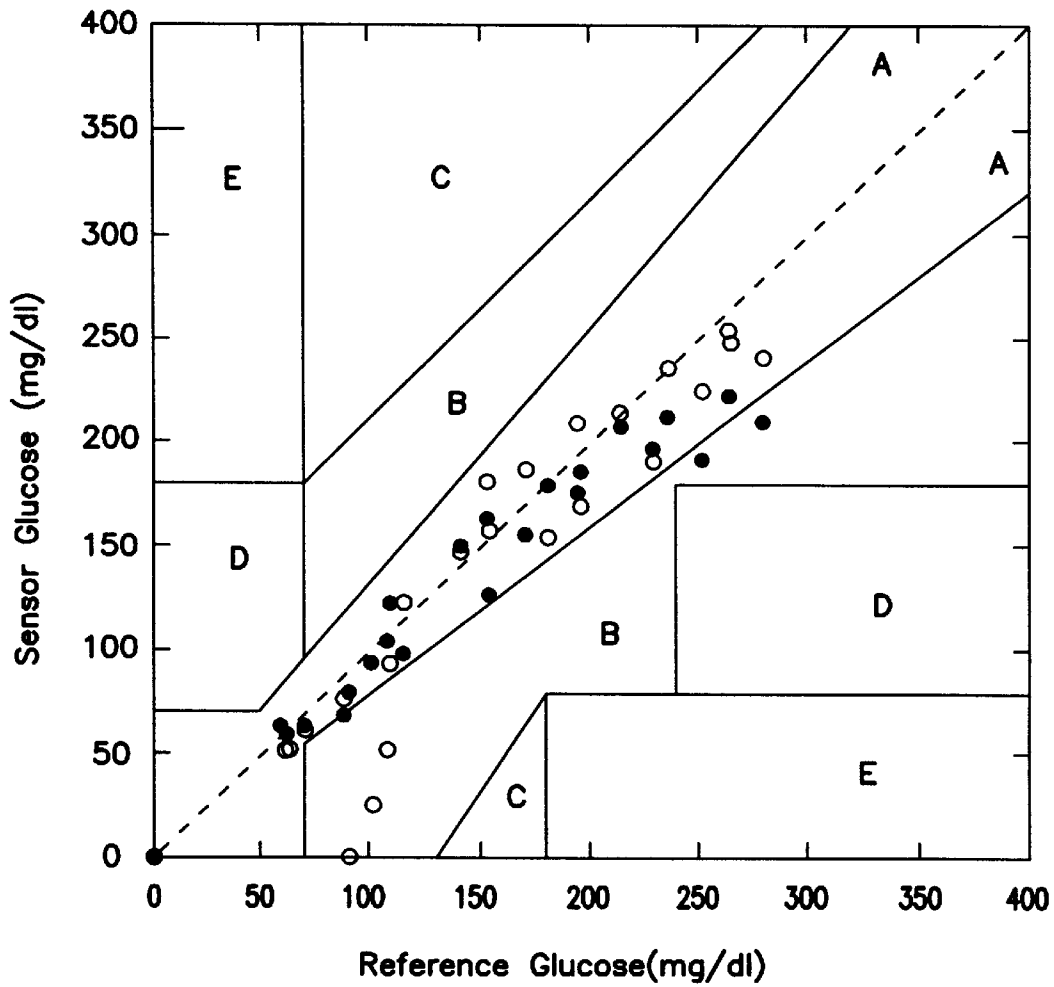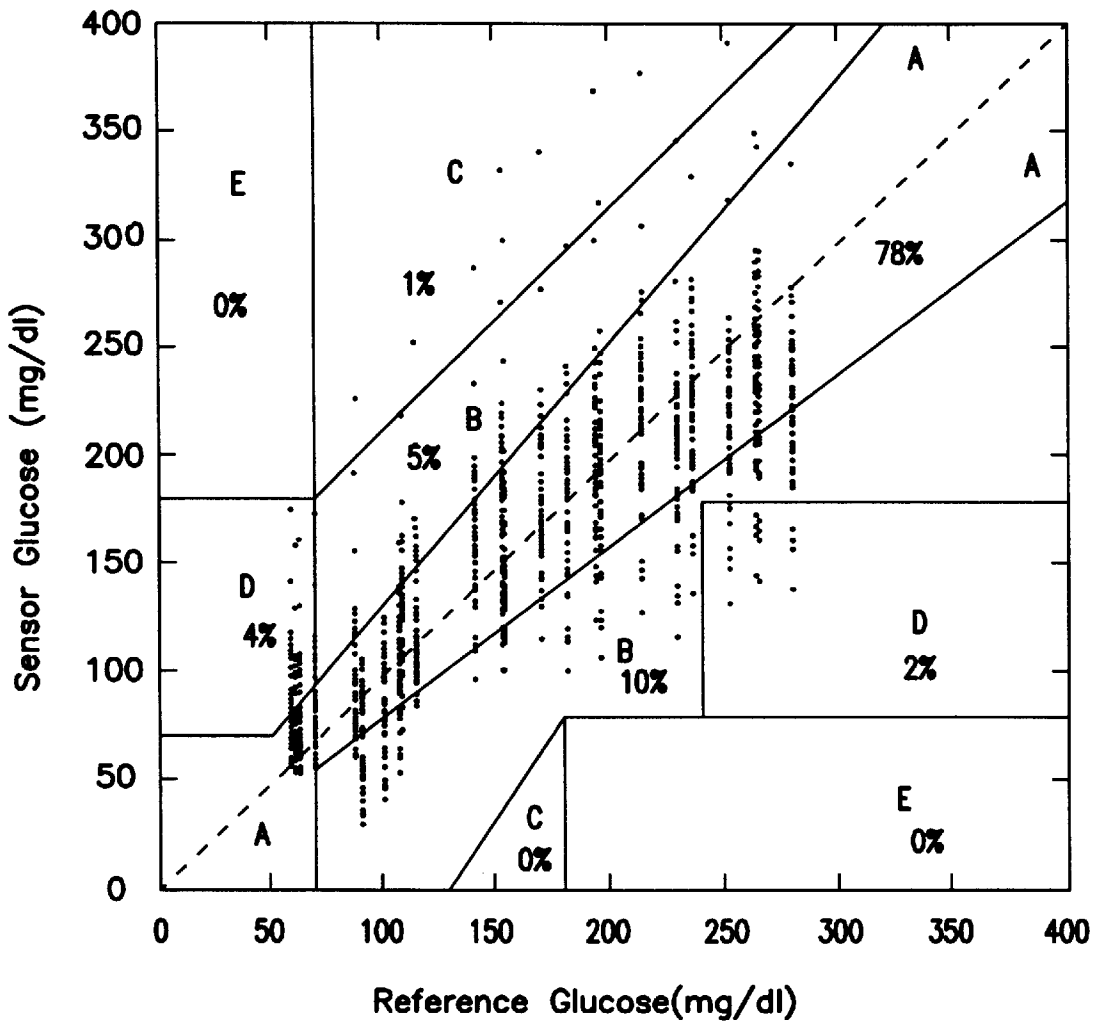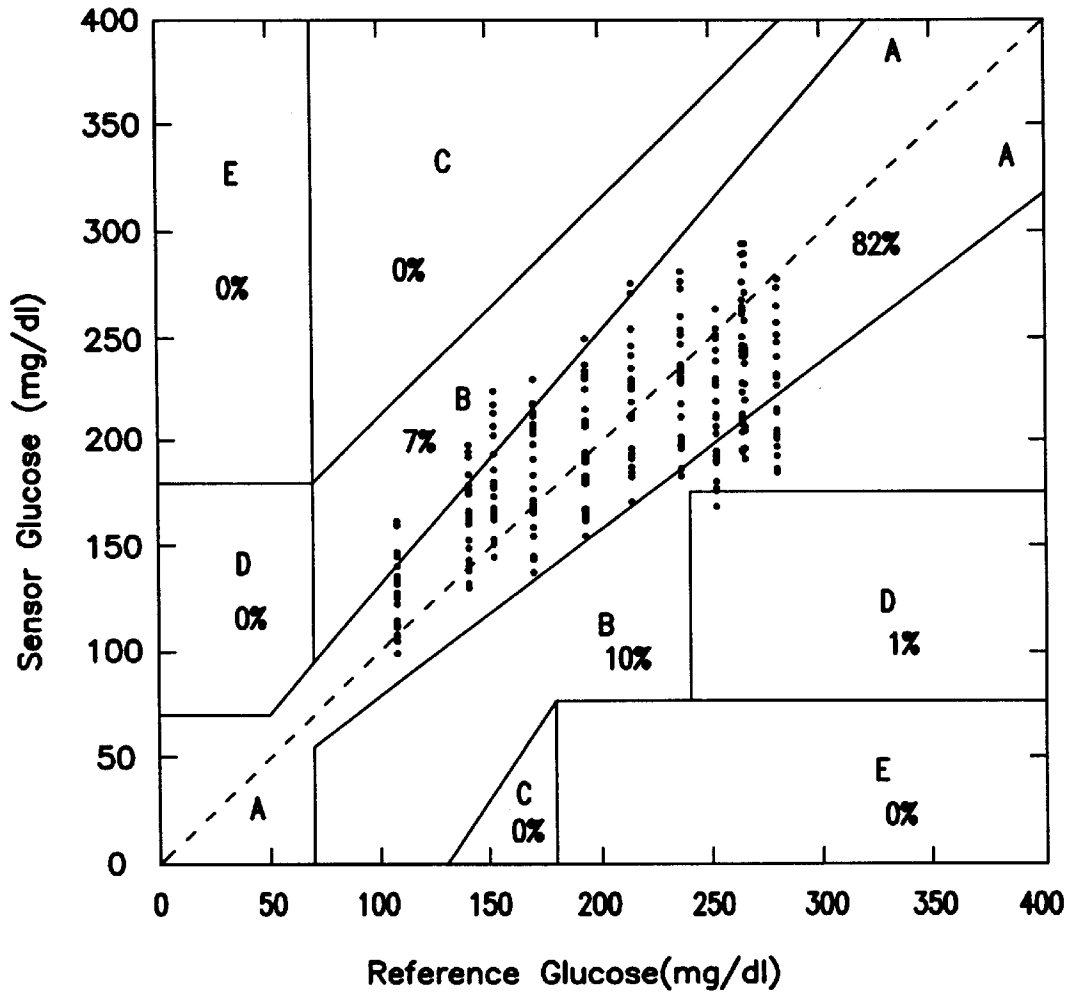
FIG. 7

FIG. 8

FIG. 9

FIG. 10

US 6,284,478 B1

1

SUBCUTANEOUS GLUCOSE ELECTRODE

This is a Continuation of application Ser. No. 08/299,
526, filed Sep. 1, 1994, now U.S. Pat. No. 5,593,852 which
application are incorporated herein by reference, which is a
continuation in part of U.S. patent application Ser. No.
08/161,682, filed Dec. 2, 1993, now U.S. Pat. No. 5,356,786,
which is hereby incorporated by reference for all purposes.

This work was supported in part by the National Insti-
tutes of Health (DK42015). Accordingly, the U.S. govern-
ment may have rights in this invention.

FIELD OF THE INVENTION

The present invention relates to in vivo enzyme biosen-
sors and more specifically to miniature glucose sensors for
subcutaneous measurement of glucose with one-point cali-
bration.

BACKGROUND

In response to the need for frequent or continuous in vivo
monitoring of glucose in diabetics, particularly in brittle
diabetes, a range of possible in vivo glucose electrodes have
been studied. The desired characteristics of these electrodes
include safety, clinical accuracy and reliability, feasibility of
in vivo recalibration, stability for at least one hospital shift
of eight hours, small size, ease of insertion and removal, and
a sufficiently fast response to allow timely intervention. The
in vivo recalibration should be based upon withdrawal of a
single sample of body fluid, e.g., blood, and measuring its
glucose concentration. This is termed "one point calibra-
tion".

Keys to safety are absence of leachable components,
biocompatibility, and limiting of the potentially hazardous
foreign matter introduced into the body to an amount that is
inconsequential in a worst case failure. The clinical accuracy
must be such that even when the readings are least accurate,
the clinical decisions based on these be still correct. Feasi-
bility of prompt confirmation of proper functioning of the
sensors and of periodic in vivo recalibration is of essence if
a physician is to allow the treatment of a patient to depend
on the readings of the sensor. This one-point calibration,
relying on the signal at zero glucose concentration being
zero and measuring the blood glucose concentration at one
point in time, along with the signal, is of essence, but has
heretofore been elusive. The sensitivity must be sufficiently
stable for the frequency of required in vivo recalibration to
not be excessive. The sensor must be small enough to be
introduced and removed with minimal discomfort to the
patient and for minimal tissue damage. It is preferred that the
sensor be subcutaneous and that it be inserted and removed
by the patient or by staff in a physician's office. Finally, its
response time must be fast enough so that corrective
measures, when needed, can be timely.

In response to some of these needs, needle type and other
subcutaneous amperometric sensors were considered. The
majority of these utilized platinum-iridium, or platinum
black to electrooxidize H_2O_2 generated by the glucose
oxidase (GOX) catalyzed reaction of glucose and oxygen. In
these sensors, the GOX was usually in large excess and
immobilized, often by crosslinking with albumin and glut-
araldehyde. To exclude electrooxidizable interferants, mem-
branes of cellulose acetate and sulfonated polymers includ-
ing Nafion™ were used. Particular attention was paid to the
exclusion of the most common electrooxidizable interfer-
ants: ascorbate, urate and acetaminophen. Also to cope with
the interferants, two-electrode differential measurements

2

were used, one electrode being sensitive to glucose and
electrooxidizable interferants and the other only to interfer-
ants. One strategy for overcoming the problem of
interferants, applicable also to the present invention,
involves their preoxidation. Another strategy involves
shifting, through chemical changes, the redox potential of
the polymer in the sensing layer to more reducing potentials.
When the redox potential of the polymer is in the region
between about −0.15 V and +0.15 V versus the standard
calomel electrode (SCE), and the electrodes are poised in
their in vivo operation between about −0.10 and +0.25 V, the
rate of electrooxidation of interferants such as ascorbate,
urate, and acetaminophen is very slow relative to that of
glucose through its physiological concentration range. Thus,
also the currents from electrooxidation of interferants are
small relative to those of glucose.

To make the electrodes more biocompatible, hydrophilic
polyurethanes, poly(vinyl alcohol) and polyHEMA mem-
branes have been used.

Several researchers tested GOX-based glucose sensors in
vivo and obtained acceptable results in rats, rabbits, dogs,
pigs, sheep and humans. These studies validated the subcu-
taneous tissue as an acceptable glucose sensing site. Good
correlation was observed between intravascular and subcu-
taneous glucose concentrations. They also demonstrated the
need for in vivo sensor calibration. Another approach to in
vivo glucose monitoring was based on coupling subcutane-
ous microdialysis with electrochemical detection. To control
and adjust the linear response range, electrodes have been
made glucose-diffusion limited, usually through glucose
transport limiting membranes.

Diffusional mediators, through which the O_2 partial pres-
sure dependence of the signals is reduced, are leached from
sensors. Such leaching introduces an unwanted chemical
into the body, and also leads to loss in sensitivity, particu-
larly in small sensors. In microsensors, in which outward
diffusion of the mediator is radial, the decline in sensitivity
is rapid. This problem has been overcome in "wired"
enzyme electrodes, i.e., electrodes made by connecting
enzymes to electrodes through crosslinked electron-
conducting redox hydrogels ("wires"). Glucose oxidase has
been "wired" with polyelectrolytes having electron relaying
$[Os(bpy)_2Cl]^{+/2+}$ redox centers in their backbones. Hydro-
gels were formed upon crosslinking the enzyme and its wire
on electrodes. These electrodes had high current densities
and operated at a potential of 0.3V vs. SCE. The electrooxi-
dizable interferants are eliminated through peroxidase-
catalyzed preoxidation in a second, nonwired, hydrogen
peroxide generating layer on the "wired" enzyme electrode.

SUMMARY OF THE INVENTION

A small (e.g., 0.29 mm), recessed, non-corroding metal
(e.g., gold, platinum, palladium) or carbon wire electrode for
subcutaneous in vivo glucose monitoring, approaching in its
performance all of the above listed requirements, including
in vivo one-point calibration, has been produced. The elec-
trode was constructed by depositing active polymer layers
into a recess formed by etching away gold from an insulated
gold wire.

The active polymer layers, including a sensing layer, a
glucose flux-limiting layer, a biocompatable layer, and
optionally a peroxidase-based interferant eliminating layer,
were protected within the recess against mechanical dam-
age. (The peroxidase-based interferant eliminating layer is
not required when a lower redox potential polymer is used,
as described above.) The recess and its polymer layers also

US 6,284,478 B1

<table>
<tr><td>3</td><td>4</td></tr>
</table>

reduced the transport of glucose to the wire electrode contacting sensing layer.

By limiting the glucose flux, the desired linear response range, spanning the clinically relevant glucose concentration range was obtained. The inventive biosensors are able to accurately measure, for example, approximately 2–30 mμ glucose and approximately 0.5–10 mμ lactate, in vivo. The sensor has no leachable components, and its four crosslinked polymer layers contain only about 5 μg of immobilized material, and only a few nanograms of polymer-bound osmium. Preoxidation of the interferants in one of the four layers makes possible one-point in vivo calibration of the sensor.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a schematic drawing of an electrode of the present invention.

FIG. 2 is a graphical representation of data generated comparing current density of glucose electrooxidation on electrodes made with PVI$_5$-Os (open triangles) with those made with PVI$_3$-Os (filled triangles).

FIG. 3 is a graphical representation of data generated comparing dependency of current generated on the depth of the recess.

FIG. 4 is a graphical representation of data generated comparing dependency of the ratio of the current generated and the charge required to electoreduce or oxidize the polymer redox centers in the sensing layer on the thickness of the sensing layer.

FIG. 5 is a graphical representation of data generated comparing variation of current generated by electrodes having sensing layers of differing thickness and diffusion limiting layers of different compositions and thickness. Solid circles: 7.5 μm thick sensing layer of PVI$_5$-Os (52%), rGOX (35%), PEGDGE (13%), coated with 4 μm PAL/PAZ (1:1 ratio). Open circles: 5.0 sensing layer. Solid triangles: 12.5 μm sensing layer and 7 μm PAL/PAZ (1:2 ratio). Open triangles: 7.5 μm sensing layer and 4.5 μm PAL/PAZ (1:2 ratio).

FIG. 6 is a graphical representation of data generated comparing dependency of current generated on the presence of ascorbate, in the absence and presence of lactate and glucose. The concentrations of ascorbate (A), lactate (L) and glucose (G) are shown. Ascorbate is an electrooxidzable interferant. Upon addition of lactate its electrooxidation current is suppressed while that of glucose is not suppressed.

FIG. 7. is a graphical representation of data showing current density and corresponding subcutaneous glucose concentration measured with the subcutaneously implanted electrodes of the present invention in a rat animal model. Large solid circles show blood glucose concentrations measured on withdrawn blood samples using a YSI analyzer.

FIG. 8 is a Clarke-type clinical grid analyzing the clinical relevance of the blood glucose measurements of FIG. 7.

FIG. 9 is a Clarke-type clinical grid of all possible correlations obtained when each of the 24 glucose analyses of FIG. 7 were used for single point calibration of either implanted electrode.

FIG. 10 is a Clarke-type clinical grid testing improvement of the single point calibration through redundant electrodes, the readings of which were within the standard deviation calculated for all differences between simultaneous readings by a pair of implanted electrodes.

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes an insulated, non-corroding conducting metal (e.g., gold, platinum, palladium)

or carbon wire-based small (e.g., 290 μm) O.D. subcutaneous glucose sensor, allowing one-point calibration in vivo. As shown in FIG. 1, its construction involves coating a small (e.g., 250 μm) diameter non-corroding metal or carbon wire 2 with an electrically insulating material 4, e.g., a polyimide, and, layering in a recess 6 formed by etching or removing a portion of the metal or carbon, the following active polymeric layers: an immobilized, "wired," glucose oxidase layer 8; an electrically insulating and glucose diffusion limiting layer 10 formed, for example, by crosslinking a polyallylamine (PAL) with a polyaziridine (PAZ); optionally, an interference eliminating layer 12, e.g., of crosslinked horseradish-peroxidase and lactate oxidase; and a biocompatible film 14 e.g., of poly(ethylene oxide) (PEO) derivatized to allow its photo-crosslinking. The outside diameter a of the wire 2 is preferably about 0.25 mm or less, and the outside diameter b of the insulated wire is preferably about 0.3 mm or less. The recess 6 in the insulated electrode extends from the tip 16 of the electrode which is open to the surrounding environment, to the top 18 of the wire 2 in the insulating sheath, generally for a length c of less than about 0.150 mm, and preferably about 0.125 mm.

The electrodes have no leachable components. The total amount of polymers and enzymes is preferably about 5 μg. The glucose response through the physiologically relevant 2–20 mM concentration range is close to linear. The electrodes do not respond to ascorbate, urate or acetaminophenol for at least about 36 hours. Their 10–90% response time is about 90 seconds at 2 mM glucose and about 30 seconds at 20 mM glucose. Their sensitivity, after about 30 minutes equilibration, is stable for about 72 hours at 37° C. in 10 mM glucose, the current deviating from the average by less than ±5%. The electrodes have substantially no signal output, e.g., current, charge, or potential, when the concentration of the analyte to be measured is zero.

Two electrodes implanted subcutaneously in a rat tracked blood glucose levels, and their absolute, uncorrected current output was proportional to the blood glucose concentration. Analysis of the correlation between the blood glucose levels in the tail vein and the current output of the sensors in the subcutaneous regions of the thorax and between the scapulae of the same rat showed that even when the probed sites and organs differed in the extreme, one point in vivo calibration was valid. The analysis also showed the value of implanting redundant sensors. Had clinical decisions been made based on individual sensor readings, calibrated at one point, 94% would have been clinically correct. By using redundant sensors and accepting only those pairs of readings that were within one standard deviation, the percentage of the clinically correct decisions was increased to 99%.

It is understood that one of skill in the art may substitute various components of the biosensor described above with known materials to obtain an modified biosensor using the principles outlined herein. For example, the following substitutions are contemplated:

Base Electrode

The base electrode of the inventive sensor may be formed of a non-corroding metal or carbon wire, for example vitreous carbon, graphite, platinum, palladium, or gold. Gold is preferred, and is used in the following illustrative examples of the invention.

Insulator

The conductive metal or carbon wire is coated with an electrically insulating material, which also forms a wall

US 6,284,478 B1

5

about the recess which houses the active polymeric components. The insulating material may be, for example, polyurethane, teflon (fluorinated polymers), polyethylene-terephthalate (PET, Dacron) or polyimide. The insulating material is preferably a biocompatible polymer containing less than about 5% water when in equilibrium with physiological body fluids, e.g., subcutaneous tissue.

Recess

In general, the recess at the tip of the electrode is approximately 20 to 150 μm in length c, and preferably is approximately 50 to 125 μm.

Etching Method

The method for etching metal from the tip of the electrode described herein may utilize chloride, bromide or iodide in the bath in lieu of cyanide as described. Bromide is preferred, because it is less toxic and, like $Au(CN)_2^-$, $AuBr_4^-$ is a water soluble anion. Thus, in aqueous HBR, the metal, e.g., gold, an be etched by applying a sufficiently oxidizing potential where gold is electrolytically dissolved:

$$Au+4\ HBr \rightarrow HAuBr_4 + 3/2\ H_2$$

Wired Enzyme Layer

In the sensing enzyme-containing layer, glucose oxidase may be substituted with other redox enzymes to measure other relevant clinical compounds. For example, lactate oxidase may be used for the in vivo detection of lactate, important in determining if an organ is receiving sufficient oxygen through the blood.

Useful redox polymers and methods for producing the sensing layer are described, for example, in U.S. Pat. Nos. 5,264,104; 5,356,786; 5,262,035, and 5,320,725. Additional redox polymers include, for example, poly(1-vinyl imidazole); poly(4-vinyl pyridine); or copolymers of 1-vinyl imidazole such as poly (acrylamide co-1-vinyl imidazole) where the imidazole or pyridine complexes with $[Os\ (bpy)_2 Cl]^{+/2+}$; $[Os\ (4,4'\text{-dimethyl bipyridine})_2Cl]^{+/2+}$; $[Os\ (4,4'\text{-dimethyl phenanthroline})_2Cl]^{+/2+}$; $[Os\ (4,4'\text{-dimethyoxy phenanthroline})_2Cl]^{+/2+}$; and $[Os\ (4,4'\text{-dimethoxy bipyridine})_2Cl]^{+/2+}$; to imidazole rings. The imidazole compounds are preferred because their complexes have more reducing redox potentials, i.e., closer to that of the SCE potential. At these more reducing potentials, the rate of electrooxidation of interferants and the current generated thereby.

Barrier Layer

The polymeric barrier layer is electrically insulating and limits diffusion of glucose through to the sensing layer. It may be formed, for example, by crosslinking a polyallylamine (PAL) with a polyaziridine (PAZ). Alternatively, PAL may be replaced wholly or in part with a zwitterionic polymer obtained by quaternizing poly(vinylpyridine) with bromoacetate and dialyzing against 0.15M NaCl or by a polyanion such as a polysulfonic acid.

The barrier layer may contain a polyanionic polymer, in which the rate of permeation of anionic interferants such as ascorbate and urate is slowed. This layer may also contain a polycation that enhances the retention of the polyanion by electrostatic bonds and improves wetting by the biocompatable layer.

Interference Eliminating Layer

As described above, this layer is optional, in that it is not required when a redox polymer having a more reducing

6

potential is used, such as PVI_{15}-dmeOs (Ohara et al., *Analytical Chemistry*, 1994, 64:2451–2457). At operating potentials of approximately –0.10 to +0.25 for the glucose biosensor, the rate of electrooxidation of interferants such as ascorbate, urate and acetaminophen is very slow relative to that of glucose through its physiological concentration range.

When a separate interferant eliminating layer is used, it preferably contains a peroxidase enzyme which may or may not be preactivated. Such interferant eliminating layers are disclosed, for example, in U.S. Pat. No. 5,356,786, which discloses the structure and function of interferant eliminating biosensors. The glucose biosensor preferably contains lactate oxidase (LOX) in combination with peroxidase in the interferant eliminating layer. However, for biosensors used to detect lactate, glucose oxidase would be used with peroxidase. In a similar manner, the enzyme composition of the interferant eliminating layer may be altered for a specified function.

Biocompatable Layer

In general, the biocompatable layer is comprised of hydrogels, e.g., polymeric compositions which contain more than about 20% by weight of water when in equilibrium with a physiological environment such as living tissue or blood. An example is crosslinked poly(ethylene oxide), e.g., poly (ethylene oxide) tetraacrylate. The polymeric compositions must be non-toxic and compatible with living systems.

Method for Making Multi-Layered Recessed Biosensors

Insulated non-corroding metal or carbon wires that have been etched as described above to contain a recess at the tip, are placed in a block that serves as an X-Y positioner. The wires vertically traverse the block and are held in place, e.g., by pressure. The blocks with the wires can be formed of elements, each element having multiple half-cylinder grooves running vertically. The wires are placed in these grooves and the elements are assembled into the block using screws. For example, the block may be formed of aluminum having equally spaced holes, (900 for a 30×30 array of wires), each hole to contain one wire. The block is positioned under a fixed micronozzle that ejects a fluid in to the recess of the insulated wire.

To reduce the requirement of precision in the positioning of the block and the micronozzle, the nozzle is electrically charged, with the wire having an opposite charge, or the wire being grounded or at least having a potential such that there is a potential difference between the nozzle and the wire. Because the nozzle is charged, the microdroplets it ejects are also charged with the same type of charge (positive or negative) as the nozzle. The higher the potential on the nozzle (e.g., versus ground potential), the higher the charge on the ejected microdroplets. If the tip of the wire to be coated is at ground potential or has a charge of the opposite type, the charged microdroplets are guided into the recess to deposit on the electrode, even if the jet of microdroplets is not vertical, i.e., even if the micronozzle is not precisely aligned above the wire's tip.

Furthermore, the higher the electrical potential on the nozzle (relative to ground) the greater the charge on the ejected microdroplet. When the charge is high enough, the droplet breaks up into two or more smaller droplets because of electrostatic repulsion of charges on the droplet. Thus, the very small droplets all "drift" (drift meaning transport assisted by an electrical field) to the recessed electrode

US 6,284,478 B1

7   8

surface and are collected on it, even if they did not originate in a nozzle precisely aligned with the electrode.

This coating method is useful in making any small biosensor, not only those in recessed zones.

Clinical Use of the Recessed Biosensors

The recessed biosensors of the present invention have sufficient sensitivity and stability to be used as very small, subcutaneous biosensors for the measurement of clinically relevant compounds such as glucose and lactate. The electrodes accurately measure glucose in the range of about 2–30 μM and lactate in the range of about 0.5–10 mM. One function of the implanted biosensor is to sound an alarm when, for example, a patient's glucose concentration is too low or too high. When pairs of implanted electrodes are used, there are three situations in which an alarm is triggered: low glucose concentration, high glucose concentration; sensor malfunction as determined by a discrepancy between paired readings of the two sensors. A discrepancy sufficient to trigger the alarm may be, for example more than two or three times the standard deviation persisting for a defined period, e.g., not less than ten minutes. Such a system may be useful in sleeping patients, and also in emergency and intensive care hospital rooms, where vital functions are continuously monitored.

Another function of the inventive biosensors in to assist diabetics in maintaining their blood glucose levels near normal. Many diabetics now maintain higher than normal blood glucose levels because of danger of coma and death in severe hypoglycemia. However, maintaining blood glucose levels substantially, e.g., approximately 40% or more above normal leads to retinopathy and blindness as well as to kidney failure. Use of the subcutaneous biosensors to frequently, if not continuously, monitor glucose concentrations is desirable so that glucose concentrations can be maintained closer to an optimum level.

The subcutaneous biosensors can be used to measure the rate of rise and decline of glucose concentrations after a meal or the administration of glucose (e.g., a glucose tolerance test). The sensors are also useful in feedback loops for automatic or manually controlled maintenance of glucose concentrations within a defined range. For example, when used in conjunction with an insulin pump, a specified amount of insulin is delivered from the pump if the sensor glucose reading is above a set value.

In all of these applications, the ability to promptly confirm that the implanted sensor reading is accurate is essential. Prompt confirmation and rapid recalibration are possible only when one-point calibration is valid. Generally, even if a sensor's response is linear through the relevant concentration range, calibration requires at least two blood or fluid samples, withdrawn from the patient at times when the glucose concentration differs. It usually takes several hours for the glucose concentration to change sufficiently to validate proper functioning by two-point calibration. The ability to confirm and recalibrate using only one point is thus a highly desirable feature of the present invention.

Redundant sensors (e.g., at least two) are preferred in clinical application of the subcutaneous biosensors. Such redundancy permits signaling of failure of any one sensor by recognition of an increase in the discrepancy between the readings of the sensors at one time point, e.g., more than two standard deviations apart. The redundant sensors may be implanted near each other or at remote sites.

It is preferred that the biosensors be implanted in subcutaneous tissue so as to make the sensor relatively unobtrusive, and at a site where they would not be easily dislodged, e.g., with turning or movement. It is also preferred, when readings are not corrected for temperature (which they generally are) that the sensors be implanted where they are likely to be at body temperature, e.g., near 37° C., and preferably covered by clothing. Convenient sites include the abdomen, inner thigh, arm.

Although we describe here continuous current measurement for assaying glucose, the electrical measurement by which the glucose concentration is monitored can be continuous or pulsed. It can be a current measurement, a potential measurement or a measurement of charge. It can be a steady state measurement, where a current or potential that does not substantially change during the measurement is monitored, or it can be a dynamic measurement, e.g., one in which the rate of current or potential change in a given time period is monitored. These measurements require at least one electrode in addition to the sensing electrode. This second electrode can be placed on the skin or can be implanted, e.g., subcutaneously. When a current is measured it is useful to have a potentiostat in the circuit connecting the implanted sensing electrode and the second electrode, that can be a reference electrode, such as an Ag/AgCl electrode. When a current is measured the reference electrode may serve also as the counter electrode. The counter electrode can also be a separate, third electrode, such as a platinum, carbon, palladium or gold electrode.

In addition to implanting the sending electrode in the body, fluid from the body, particularly fluid from the subcutaneous region, can be routed to an external sensor. it is preferred in this case to implant in the subcutaneous region a microfiltration giver and pull fluid to an evacuated container, the fluid traversing a cell containing the sensing electrode. Preferably this cell also contains a second electrode, e.g., a reference electrode which may serve also as a counter electrode. Alternatively, the reference and counter electrodes may be separate electrodes. In coulometric measurements only two electrodes, the sensing electrode and the counter electrode are required. The flow of body fluid amy be pulsed or continuous. Other than an implanted microfiltration fiber, also a microdialysis fiber may be used, preferably in conjunction with a pump.

Increased Stability of the Biosensors

To increase the stability and useful life of the inventive biosensors, it is advantageous to use intrinsically more stable enzymes and redox polymers. However, even if the enzyme and redox polymer degrade in the glucose electrooxidation process by which the signal (current) is generated, it is possible to greatly extend the useful life of the implanted electrodes and reduce the frequency of their required recalibration after implantation.

A simple measure by which the life of the implanted electrodes can be extended and the frequency of their required recalibration reduced involves turning the electrodes "on" by applying a bias, i.e., a potential, only during the period of measurement, then turning the biasing potential off or reducing it, so that a lesser current will flow. It is generally sufficient to perform only one measurement every five or even ten minutes, or longer, because glucose concentrations do not change abruptly.

Another measure is to lower the glucose flux to the sensing layer much as possible, consistent with maintaining adequate sensitivity and detectivity. Reduction of the glucose flux to the sensing layer reduces the current. Therefore, even though this stabilizes the electrodes, i.e., slows the loss

US 6,284,478 B1

9

in sensitivity, the flux dependent current must not be excessively reduced. Usually a current of 3–5 nA at 2 mM glucose concentration is adequate. When the glucose flux is lowered by using one or more glucose-flux reducing polymer slayers, such as the PAL/PAZ layer, the lifetime of the sensor is increased.

EXAMPLES

Example 1

Electrode Preparation

Electrodes were made of a polyamide-insulated 250 μm diameter gold wire, having an outer diameter (O.D.) of 290 μm (California Fine Wire Co., Grover City, Calif.). Heat shrinkable tubing (RNF 100 ³⁄₆₄– BK and ¹⁄₁₆" BK, Thermofit®, Raychem, Menlo Park, Calif.) and a two component silver epoxy (Epo-tek H₂OE; Epoxy Tech, Inc., Billerica, Mass.) were used for electrode preparation.

The glucose sensing layer was made by crosslinking a genetically engineered glucose oxidase (rGOX) (35% purity, Chiron Corp., Emeryville, Calif.) with a polymer derived of poly(vinylimidazole) (PVI), made by complexing part of the imidazoles to $[Os(bpy)_2Cl]^{+/2+}$. The resulting redox polymer, termed PVI-Os, was synthesized according to a previously published protocol. (Ohara et al., 1993, *Anal. Chem.*, 65:24). Poly(ethylene glycol) diglycidyl ether 400 (PEDGE; Polysciences, Warrington, Pa.) was used as the crosslinker.

The barrier layer between the sensing and interference-eliminating layers was made of polyallylamine (PAL; Polysciences) crosslinked with a polyfunctional aziridine (PAZ) (XAMA-7; Virginia Chemicals, Portsmouth, Va.).

The interference-eliminating layer was prepared by co-immobilizing horseradish peroxidase (HRP) type VI (Cat. no. P-8375, 310 U/mg, denoted herein as HRP-VI, Sigma, St. Louis, Mo.) and HRP for immunological assay (No. 814407, min 1000 U/mg, denoted HRP-BM, Boehringer-Mannheim, Indianapolis, Ind.) with lactate oxidase from *Pediococcus sp. (Cat. No.* 1361, 40 U/mg denoted LOX, Genzyme, Cambridge, Mass.) and a recombinant microbial source (Cat. No. 1381 denoted rLOX, Genzyme). Co-immobilization was performed using sodium periodate (Cat. No. S-1147, Sigma) according to the methods described in Maidan and Heller, 1992, *Anal. Chem.* 64:2889–2896.

The biocompatible layer was made of 10% aqueous poly(ethylene oxide) tetraacrylate (PEO-TA). To form the photocrosslinkable polymer, PEO was acrylated by reaction with acryloyl chloride. The 18,500 g/mol PEO (Polysciences) is a tetrahydroxylated compound by virtue of two hydroxyl groups on a bisphenol A bisepoxide that linked two α, ω-hydroxy-terminated 9,000 g/mol PEO units. Acryloyl chloride (Aldrich, Milwaukee, Wis.) in a 2 to 5 molar excess was used to acrylate the polymer (10% w/v PEO in benzene). Triethylamine (Mallinkrodt, Paris, Ky.) was used as a proton acceptor equimolar with theacryloyl chloride.

Other chemicals used were bovine serum albumin (BSA) fraction V (Cat. No. A-2153), BSA, ascorbic acid, uric acid, 4-acetaminophenol, L(+)=lactic acid, and hydrogen peroxide 30%, all from Sigma. All chemicals were used as received. Solutions (if not otherwise specified) were made with distilled, deionized water. Glucose monitoring was performed in buffer, in bovine serum (Sigma, Cat. No. S-6648) containing antibiotic-antimycotic solution (Sigma, Cat. No. A-8909) at 37° C. and in rats.

10

Instrumentation

In making the recessed gold electrodes, a potentiostat/galvanostat (PAR Model 173, Princeton Applied Research, Princeton, N.J.) operated in a galvanostatic mode, and a sonicator (Fisher Scientific, Pittsburgh, Pa.) were used. Cyclic voltammograms were recorded with a potentiostat (PAR Model 273A) and a conventional electrochemical cell having a Pt wire counter and a SCE reference electrode and were evaluated with PAR 270 software. Glucose signals were monitored with a bipotentiostat (Biometra EP 30) and a two channel strip-chart recorder. The recessed electrodes were coated under a microscope (Bausch & Lomb) using a micromanipulator (Narishige, Seacliff, N.Y.). The micropipettes were pulled with a micropipette puller (Narishige). Temperature was controlled with an isothermal circulator (Fisher Scientific).

Electrode Preparation

Five cm lengths of polyamide insulated gold wire were cut with a sharp razor blade. Electrical contact was made at one end with silver epoxy to an insulated stainless steel wire and the junction was covered with insulating heat shrinkable tubing. The recess forming electrochemical etching process was carried out in 10 ml of 3M potassium cyanide, with the gold wire as the working electrode and a platinum or gold wire as the counter electrode. The wires were placed in contact with the bottom of the beaker, all electrodes being equidistant from the counter electrode. The beaker was sonicated during the etching procedure. The ends of the gold wires were bent upwards, so that agitation by the sonicator caused the oxygen bubbles formed during the etching process to rise and escape. The electrodes were then thoroughly washed and immersed in water for 30 minutes.

A recess **6**, i.e., channel, in a polyamide insulated gold wire **2** is formed by electrochemical etching of the gold under galvanostatic control. By controlling the charge, the total amount of gold electrooxidized and dissolved as $Au(CN)_2$ is defined. When the conditions were set so that the CN- transport into the channel and the $Au(CN)_2$- transport out of it are not rate limiting, (e.g., sonicated bath and high concentration of potassium cyanide, at least approximately 0.2M, and preferably 3M), a flat gold wire surface is produced at the bottom of channels with aspect ratios of 0.5 to 2.0. Thus, when the CN-concentration is high enough and the wires are ultrasonically vibrated, the tips of gold wires are flat. Passage of 1.5 coulombs per electrode at 8 mA current produced approximately 125 μm deep cavities or channels. At theoretical efficiency for one-electron oxidation, 3.08 mg of gold would have been etched. The amount of gold actually etched was only 0.076 mg, showing significant CN- or water oxidation.

Nevertheless, the process is reproducible, accurate and fast with 20 electrodes being processed in each batch in less than five minutes. The recess-forming procedure was highly reproducible, with a deviation of ±10 μm found (using an objective micrometer) for a batch of 30 recessed electrodes. Before coating, the electrodes were examined under a microscope for flatness of the gold surface and correct depth.

FIG. 1 shows a schematic side view in cross-section of an electrode of the present invention, showing the gold wire **2**, insulating coating **4**, and recess or channel **6**. The recessed gold surfaces were coated by filling of the cavities or channels **6** with aqueous solutions containing the crosslinkable components of the different layers, and their crosslinkers. The solutions were introduced under a microscope with a micropipette (connected to a microsyringe by polyethylene

US 6,284,478 B1

11

tubing and shrink tubing), using a micromanipulator. After application of each of the individual layers, the electrodes were cured overnight at room temperature, in air.

Electrode Structure

The electrodes were prepared by sequentially depositing four layers within the recess or channel **6**. The layers were: the sensing layer **8**, the insulating layer **10**, the interference-eliminating layer **12** and the biocompatible layer **14**. The sensing layer, containing "wired" redox enzyme is positioned adjacent to and in contact with the gold wire **2**. The insulating layer **10** is positioned between the sensing layer **8** and the peroxidase-based interferant-eliminating layer **12**. The biocompatible layer **14** fills the remaining space in the recess **6** and is in contact with the environment outside the electrode. The thin polymer layers are well protected by containment within the polyamide sleeve **4**.

The sensing layer **8** was made by "wiring" rGOX to the gold electrode through a redox hydrogel to which the enzyme was covalently bound. The electrodes were prepared as follows: 10 mg/ml solutions were made from

1. the PVI-Os redox polymer in water,
2. the crosslinker, PEGDGE, in water, and
3. the enzyme, rGOX, in a 10 mM HEPES solution adjusted to pH 8.15.

A redox hydrogel was formed by mixing the three solutions so that the final composition (by weight) was 52% redox polymer, 35% enzyme and 13% crosslinker.

The insulating layer **10** prevented electrical contact between the redox hydrogel and the interference eliminating enzymes (HRP and LOX). PAL:PAZ was used as the insulating material. The film was deposited from a solution obtained by mixing in volume ratio of 1/1, 1/2 or 1/3, a PAL solution (4.5 mg in 100 mM HEPES buffer at pH 7.0) and a freshly prepared PAZ solution (30 mg/ml). The PAZ solution was used within 15 minutes of preparation.

The interference-eliminating layer **12** was prepared according to a previously published protocol, Maidan and Heller, 1992, *Anal. Chem.*, 64:2889–2896. 50 μl of a 12 mg/ml freshly prepared sodium periodate solution was added to 100 μl of a solution containing 20 mg/ml HRP (HRP-VI or HRP-BM) and 100 mg/ml LOX (LOX or rLOX) in 0.1 M sodium bicarbonate and the mixture was incubated in the dark for two hours. Alternatively, the oxidation of HRP could be carried out prior to adding LOX and crosslinking.

The biocompatible layer **14** films were photocrosslinked by exposure to UV light (UVP, Inc., San Gabriel, Calif.; Blak-Ray; spectral peak at 360 nM, UV irradiance at the sample 200 mW/cm^2) for one minute. The initiator used was 2,2-dimethoxy-2-phenylacetophenone (Aldrich). A solution of 300 mg/ml of the initiator in 1-vinyl-2-pyrrolidinone (Aldrich) was added to the prepolymer mixtures. Approximately 30 μl of the initiator solution was added per ml of 10% w/w aqueous solution of the tetraacrylated PEO. The prepolymers were crosslinked in situ inside the recess of the electrode. The films were prepared by filling the recess with the prepolymer solution twice and exposing the electrode to the UV light source after each time the cavity was filled.

In vitro Testing of Electrodes

In vitro experiments were carried out in batch fashion at 25° and 37° C., using a conventional three electrode electrochemical cell with the enzyme-modified gold wire as the working electrode, a platinum wire as the counter electrode and a saturated calomel reference electrode (SCE). The

12

electrolyte was a 20 mM phosphate buffered-saline solution containing 0.15 M NaCl at pH 7.15. Experiments in serum were performed at 37° C., adding 100 μL antibiotic-antimycotic solution to 10 ml serum. Phosphate buffered-saline and serum were agitated during the experiments. The working potential was +0.3 V versus SCE for experiments with the PVI-Os polymers.

Structure and Performance

The depth c of the channel **6** and the thickness of the polymer layers in it controls the mass transport, i.e., flux of glucose, to the sensing layer. By controlling these parameters, the apparent Michaelis constant (K_m) is adjusted to about 20–30 mM glucose. The polyimide wall **4** of the channel **6** also protects the four polymer and polymer/enzyme layers **8, 10, 12, 14** against mechanical damage and reduces the hazard of their loss in the body. Because the glucose electrooxidation current is limited by glucose mass transport through the recess **16** and its polymer films **8, 10, 12, 14**, rather than by mass transport to the tissue-exposed tip **16**, the current is practically insensitive to motion. Evidently, the electrooxidation rate of glucose in the recessed sensing layer **8** is slower than the rate of glucose diffusion to the channel's outer fluid contacting interface.

PVI_5-Os is preferred as the "wire" of the sensing layer when an interference eliminating layer of HRP and LOX is used, but not in the absence of this layer, i.e., when redox polymers with more reducing redox potential are preferred. The subscript (**5**) is used to indicate that, on the average, every fifth vinylimidazole mer carries an electron-relaying osmium center. Use of electrodes formed with PVI_5-Os and PVI_3-Os (every third 1-vinylimidazole mer carrying an osmium center) are compared in FIG. **2**, and show higher current density of glucose electrooxidation on electrodes made with PVI_5 - Os (open triangle) than on those made with PVI_3 - Os (filled triangles).

Depth of the Recess and the Sensing Layer

Channels of 125, 250, and 500 μm depth, were investigated to assess the dependence of the current on the depth of the recess (FIG. **3**), with the total amount of PVI_5 - Os and rGOX being kept constant. Much of the loss in current in the deeper cavities resulted not from reduced glucose mass transport, but from adsorptive retention of part of the enzyme and polymer on the polyamide wall when micro-drops of the component solutions were introduced into the recess in the process of making the electrodes. Through repeated rinsing with water, some of the adsorbed polymer and enzyme on the walls were washed onto the electrode surface, increasing the current. The highest currents were seen after five washings. When the thickness of the sensing layer was increased through increasing the number of coatings (FIG. **4**) the ratio of current to charge required to electroreduce or electrooxidize the redox polymer in the sensing layer reached a maximum, then dropped. For the preferred 125 μm recess, 10 coatings, producing an approximately 13 μm thick wired-rGOX sensing layer, yielded sensors that had the desired characteristics for in vivo use.

The Insulating Layer

This layer electrically insulates the redox enzymes of the interference eliminating layer (HRP and LOX) from the "wired" rGOX layer and limits the glucose flux to the sensing layer, thereby extending the useful life of the electrode. PAL crosslinked with PAZ, forming a polycationic network at pH 7.09 is preferred. The best results, i.e., best

US 6,284,478 B1

13   14

stability of current outputs, were obtained using 1:2 PAL-
:PAZ (FIG. **5**), with three coatings applied to form an
approximately 7 μm thick crosslinked film.

The Interference Eliminating Layer

Interferants, particularly ascorbate, urate, and
acetaminophenol, are oxidized in the third layer, containing
LOX and HRP. In this layer, lactate, the typical concentra-
tion of which in blood is 1 mM, reacts with O_2 to form H_2O_2
and pyruvate. H_2O_2, in the presence of HRP, oxidizes
ascorbate, urate, and acetaminophenol, being reduced to
water. The preferred coimmobilization process involved two
separate steps: periodate oxidation of oligosaccharide func-
tions of HRP to aldehydes, followed by mixing with LOX
and formation of multiple Schiff bases between HRP-
aldehydes and LOX amines (e.g. lysines) and between HRP
aldehydes and amines. The thickness of the interference
eliminating layer is approximately 85 μm and is made by
applying successive coatings, e.g., about six coatings. FIG.
6 shows that electrooxidizable interferants were eliminated
in the presence of lactate at physiological levels. LOX
slowly lost its activity in the crosslinked HRP-LOX layer.
This led to degradation of the ability of the layer to eliminate
interferants. After 36 hours of operation at 37° C., a mea-
surable current increment was noted when enough ascorbate
was added to produce a 0.1 mM concentration.

The Biocompatible Layer

A preferred biocompatible layer consists, for example, of
photocrosslinked tetraacrylated 18,500 Da poly(ethylene
oxide) (Pathak et al., 1993, *J. Am. Chem. Soc.,*
114:8311–8312). The thickness of this layer, made by
sequential photo-crosslinking of two coatings, is about 20
μm. One minute UV exposure required for the photo-
crosslinking process reduced the sensitivity by 16±2%.

Example 2

In vivo Use of Sensor

The objective of this experiment was to establish the
validity of a one-point in vivo calibration. Two sensors were
simultaneously implanted subcutaneously in a rat, one on
the thorax, the second between the scapulae. To make the
difference between the blood sampled and the subcutaneous
fluid proved with the sensors as extreme as possible, i.e., to
probe whether the one-point calibration holds even if the
organs sampled are different and the sampling sites are
remote, blood was withdrawn from the tail vein. Blood
glucose levels were periodically measured in withdrawn
samples, while the absolute uncorrected sensor current out-
put was continuously monitored.

In vivo experiments (6–10 hours) were carried out in 300
g male Sprague-Dawley rats. The rats were fasted overnight
and prior to the experiment were anaesthetized with an
intraperitoneal (i.p.) injection of sodium pentobarbital (65
mg/kg rat wt). An i.p. injection of atropine sulfate (166
mg/kg rat wt) was then administered to suppress respiratory
depression. Once the rat was anaesthetized, a portion of the
rat's abdomen was shaved, coated with a conductive gel, and
an Ag/AgCl surface skin reference electrode was attached.
This electrode served also as the counter electrode. Sensors
were then implanted subcutaneously using a 22 gauge
Per-Q-Cath Introducer (Gesco International, San Antonio,
Tex.) on the rat's thorax, or subcutaneously in the intras-
cepular area through a small surgical incision. The sensors
were taped to the skin to avoid sensor movement. The

sensors, along with the reference electrode, were connected
to an in-house built bipotentiostat. The operating potential of
the sensors was 0.3 V vs. Ag/AgCl, with the Ag/AgCl
electrode serving as both the reference counter electrode.
Sensor readings were collected using a data logger (Rustrak
Ranger, East Greenwich, R.I.) and at the end of the experi-
ment were transferred to a computer. During the experiment,
the rat's body temperature was maintained at 37° C. by a
homeostatic blanket. The sensors were allowed to reach a
basal signal level for at least one hour before blood sampling
was started. Blood samples were obtained from the tail vein
and all blood samples were analyzed using a glucose ana-
lyzer (YSI, Inc., Yellow Springs, Ohio; Model 23A).

Approximately thirty minutes after the start of blood
sampling, an i.p. glucose infusion was started using a
syringe pump (Harvard Apparatus, South Natick, Mass.) at
a rate of 120 mg glucose/min kg rat wt. The glucose infusion
was maintained for approximately one hour.

As seen in FIG. **7**, at 410 min the current dropped
precipitously. Such a drop was observed in other measure-
ments with subcutaneously implanted electrodes between
400 and 600 min, but was never observed in electrodes
operated in buffer at 37° C. When the failed electrodes were
withdrawn and retested in buffer, most of their original
sensitivity was found to be intact. The cause for this apparent
deactivation was failure of the counter/reference Ag/AgCl
electrode on the rat's skin to make good electrolytic contact,
and was not due to any failure of the implanted sensor. Using
an arbitrarily chosen point to calculate a calibration curve
for each electrode, i.e., one blood glucose level determina-
tion and one current measurement to establish the scales, all
the data from FIG. **7** were plotted in a Clarke-type, (Clarke
et al., 1987, *Diabetes Care,* 5:622–627) clinical grid (FIG.
8), without further correction. In this analysis, points falling
in region A of the grid are considered clinically accurate,
while those in region B are considered clinically correct.
Points falling in region C are not correct, but would not lead
to improper treatment. Points in regions D and E are
incorrect and if treatment would rely on these, it would be
improper.

All of the points, from both electrodes, were in regions A
and B, with 43 of the 48 points being in region A. The three
points in region B near 100 mg/dl glucose, for the electrode
implanted between the scapulae, were the last three points of
the experiment, at about 410 min. Notwithstanding the
failure mode at 400–600 min because of poor electrolytic
contact of the counter/reference electrode with the skin and
failure after 36 hours by deactivation of the lactate oxidase,
resulting in loss of interference elimination, one-point cali-
bration is shown here to be practical. After such calibration,
the readings of the subcutaneous sensors provide, without
any correction, clinically useful estimates of blood glucose
levels.

FIG. **9** shows the distribution of all possible correlations
obtained when each of the 24 glucose analyses was used for
single point calibration of either implanted electrode. There
are 2×24×24=1152 points in the distribution. Of these, 78%
are in region A, 15% are in region B, 1% in region C, 6%
are in region D, and no points are in region E.

In FIG. **10**, we tested for the improvement of the single
point calibration through using redundant electrodes. First,
the readings of electrode A were normalized with respect to
those of electrode B by multiplying each reading by the
average output of electrode B divided by the average output
of electrode A. Next the standard deviation was calculated
for the differences between the 24 sets of readings of

15

implanted electrode B and corrected readings of implanted electrode A. Then, all those sets of readings that differed by more than the standard deviation were rejected. The number of sets was reduced thereby from 24 to 11; 82% of the points were in region A, 17% in region B, 1% in region D, and no points in regions C and E. The distribution demonstrates that the sensors can be calibrated through a single independent measurement of the glucose concentration in a withdrawn blood sample. They also demonstrate the improvement in clinical accuracy resulting from the use of redundant sub-cutaneous sensors. The selection of those data points that differed by less than the standard deviation for the entire set led to a sixfold reduction in the probability of clinically erring in a decision based on readings of the implanted sensors.

Stability and Other Characteristics

In order to improve the stability, more thermostable recombinant GOX, (rGOX; Heller, 1992, *J. Phys. Chem.*, 96:3579–3587) rather than GOX is used in the sensor and glucose transport is reduced to make the sensor current diffusion, not enzyme turnover, limited. The glucose flux is attenuated by the three outer layers and the sensing layer itself. Because the sensing layer contains a large excess of glucose oxidase, its activity greatly exceeds that needed for electrooxidizing the attenuated glucose flux, and the sensor's stability is improved.

The stability can be tested by methods known, for example, tested in the presence of 0.1 mM ascorbate in 10 mM glucose at 37° C. The current output of a typical optimized electrode was about 35 nA and the apparent K_m, derived from an Eadie-Hofstee plot, was about 20 mM (Table 1). The 10–90% response time was approximately one minute.

As expected, and as can be seen in FIG. **5**, with thinner films the glucose mass transport was increased, i.e., the current was higher, while for thicker films the stability was improved. Because of the high sensitivity of thin sensing film (approximately 1 μm) electrodes (less than $10^{-2} A\,cm^{-2}\,M^{-1}$), an order of magnitude decrease in sensitivity could be traded for stability, while the currents remained high enough to be easily measured.

As seen in FIG. **5**, the sensitivity of the stabilized sensors does not change by more than ±5% for 72 hours of operation at 37° C. After a small initial decrease in sensitivity, it increased to a maximum after 40 hours and the final 72 hour sensitivity was almost identical with the initial.

The characteristics of the electrodes of the present invention are summarized in Table 1. Each entry represents an average value for five tested electrodes. Baseline currents are typically less than 0.5 nA and the noise less than 10 pA. The currents observed throughout the physiological glucose concentration range (2–20 mM) exceed the noise equivalent current by at least a factor of 100. The apparent K_m is 20 mM, and the 10% to 90% response time is, for aged electrodes, about 90 seconds at the lowest physiologically relevant glucose concentration (2 mM) and 20 seconds at the highest (20 mM).

The baseline of nil at 0 mM glucose is stable for 36 hours in the presence of 0.1 mM ascorbate. The stability observed and the existence of a valid zero-point in the presence of interferants suggest that the sensor can be used in vivo for 72 hours and tested/recalibrated in vivo through a single point calibration, i.e., by withdrawing only a single sample of blood for independent analysis.

16

TABLE 1

SENSOR CHARACTERISTICS

i(nA)	j(μA/cm^2)	K_M^{app}(mM) EH	K_M^{app}(mM) LB	t_r(s)	Current Variance (%)
33.9	69.1	18.5	33.4	30–90	5.0

where:
i is the current measured at 37° C. and at 10 mM glucose concentration
j is the current density measured at 37° C. at 10 mM glucose concentration
K_M^{app} is the apparent Michaelis-Menten coefficient determined from an electrochemical Eadie-Hoffstee (EH) of Lineweaver-Burk (LB) plot
t_r is the 10–90% risetime, 90s for 2 mM and 30 s for 20 mH glucose concentration.
Current Variance is the maximum deviation from the mean value, measured during the 72 hour test, conducted in 10 mM glucose in the presence of interferants. The current was continuously monitored at 37° C.

The foregoing examples are designed to illustrate certain aspects of the present invention. The examples are not intended to be comprehensive of all features and all embodiments of the present invention, and should not be construed as limiting the claims presented herein.

We claim:

1. An electrochemical sensor comprising:

one or more non-corroding metal or carbon electrodes;

a sensing layer comprising an enzyme coupled to each electrode; and

a biocompatible layer comprising a biocompatible hydrogel chemically bound to the sensing layer of each electrode.

2. The electrochemical sensor of claim **1**, wherein the biocompatible layer is indirectly chemically bound to the sensing layer.

3. The electrochemical sensor of claim **2**, wherein the sensor further comprises an interferent eliminating layer or an analyte flux limiting layer or both and the biocompatible layer is chemically bound to the interferent eliminating layer or the analyte flux limiting layer.

4. An analyte measurement system comprising:

an electrochemical sensor including two or more non-corroding metal or carbon electrodes, each electrode adapted for subcutaneous implantation in an animal, and a non-leachable analyte-responsive enzyme disposed on each of the electrodes; and

a device for comparing signals generated at the two or more electrodes.

5. An electrochemical sensor comprising:

one or more non-corroding metal or carbon electrode;

a sensing layer coupled to each electrode wherein the sensing layer comprises a non-leachable redox enzyme; and

a microfiltration device for transporting a fluid sample into contact with the sensing layer of at least one of the electrodes.

6. The electrochemical sensor of claim **5**, wherein the microfiltration device comprises a microfiltration fiber or a microdialysis membrane.

7. The electrochemical sensor of claim **5**, wherein the electrochemical sensor is adapted for implantation in the body of an animal, the sensor comprising a redox compound and the redox enzyme which are both non-leachable by fluids in the body of the animal at a pH between about 6.5 and about 7.8.

8. The electrochemical sensor of claim **7**, wherein the sensor is configured and arranged so that when the sensor is

US 6,284,478 B1

17

implanted in the body of an animal, only animal fluids containing the sample will be transported by the microfiltration device to the sensor.

9. An electrochemical sensor for measuring an analyte in an animal, comprising:
  one or more analyte responsive electrodes, at least one of said analyte-responsive electrodes adapted for subcutaneous implantation in an animal, each of the analyte responsive electrodes comprising
  a non-corroding metal or carbon electrode, and
  a sensing layer covering at least a portion of each non-corroding metal or carbon electrode, comprising a redox enzyme and a redox compound,
  wherein the redox enzyme and redox compound are non-leachable by fluids in the body of the animal at a pH of between about 6.5 and about 7.8.

10. The electrochemical sensor of claim 9, wherein the redox compound comprises a non-leachable redox polymer.

11. The electrochemical sensor of claim 10, wherein the redox polymer is a crosslinked redox polymer.

12. The electrochemical sensor of claim 11, wherein the redox polymer is crosslinked with the redox enzyme.

13. The electrochemical sensor of claim 9, wherein the redox compound comprises a metal ion selected from the group consisting of $Os^{3+/2+}$, $Ru^{3+/2+}$, and $Fe^{3+/2+}$.

14. The electrochemical sensor of claim 13, wherein the metal ion is $Os^{3+/2+}$.

15. The electrochemical sensor of claim 13, wherein the metal ion is $Ru^{3+/2+}$.

16. The electrochemical sensor of claim 13, wherein the metal ion is bound to a polymer.

17. The electrochemical sensor of claim 16, wherein the polymer is poly(1-vinylimidazole) or a copolymer of 1-vinylimidazole.

18. The electrochemical sensor of claim 16, wherein the metal ion is bound to the polymer by electrostatic or ionic bonding.

19. The electrochemical sensor of claim 16, wherein the metal ion is bound to the polymer by covalent bonding.

20. The electrochemical sensor of claim 16, wherein the metal ion is bound to the polymer by coordinative bonding.

21. The electrochemical sensor of claim 9, wherein the redox potential of the redox compound is not more reducing than about –0.15 V and not more oxidizing than about +0.15 V versus the standard calomel electrode in an aqueous solution at about pH 7.4.

22. The electrochemical sensor of claim 9, wherein the redox potential of the redox compound is substantially invariant in a pH range of between about 6.5 and 7.8.

23. The electrochemical sensor of claim 9, wherein the one or more non-corroding metal or carbon electrodes are flexible.

24. The electrochemical sensor of claim 9, wherein the electrochemical sensor further comprises a biocompatible layer covering the sensing layer of each non-corroding metal or carbon electrode.

25. The electrochemical sensor of claim 24, wherein the biocompatible layer comprises repeating units of ethylene oxide.

26. The electrochemical sensor of claim 9, wherein the electrochemical sensor further comprises an analyte flux limiting layer over the sensing layer of at least one of the one or more non-corroding metal or carbon electrodes.

27. The electrochemical sensor of claim 9, wherein the electrochemical sensor further comprises an electrically insulating polymer coating at least a part of one of the one or more non-corroding metal or carbon electrodes.

18

28. The electrochemical sensor of claim 27, wherein the electrically insulating polymer is selected from the group consisting of polyimide, polyester, polyurethane, and per-fluorinated polymer.

29. The electrochemical sensor of claim 9, wherein the outer diameter of each of the one or more non-corroding metal or carbon electrodes is smaller than about 0.3 mm.

30. The electrochemical sensor of claim 9, wherein the sensor further comprises an interference eliminating layer covering the sensing layer of each non-corroding metal or carbon electrode.

31. The electrochemical sensor of claim 30, wherein the interference eliminating layer comprises a peroxidase enzyme.

32. The electrochemical sensor of claim 9, wherein the sensitivity of the sensor does not change by more than about ±5% for 72 hours of operation at 37° C.

33. The electrochemical sensor of claim 9, wherein the sensitivity of the sensor does not change by more than about ±5% for 8 hours of operation at 37° C.

34. The electrochemical sensor of claim 9, wherein the sensor further comprises one or more reference or reference/counter electrodes, at least one of the reference or reference/counter electrodes adapted for placement on the surface of the animal.

35. The electrochemical sensor of claim 9, wherein each non-corroding metal or carbon electrode is substantially coated with an electrically insulating polymer and has a gap or recess in the coating exposing a bare, non-insulated surface of the electrode, the insulating polymer containing less than 5% water by weight when in equilibrium with physiological body fluids at about 37° C.

36. The electrochemical sensor of claim 35, wherein the exposed electrode is recessed in the insulating polymer coat forming a channel in the insulating polymer coat.

37. The electrochemical sensor of claim 36, wherein the channel has a depth ranging from about 20 microns to about 1 mm.

38. The electrochemical sensor of claim 9, further comprising
  one or more alarms, each alarm coupled to one or more of the non-corroding metal or carbon electrodes.

39. The electrochemical sensor of claim 38, wherein one or more of the alarms is configured to activate if a signal generated at one of the electrodes coupled to the alarm is outside a predetermined range.

40. The electrochemical sensor of claim 38, wherein at least one of the alarms is configured to activate if a signal generated at the electrode indicates a blood glucose concentration above a predetermined concentration.

41. The electrochemical sensor of claim 38, wherein at least one of the alarms is configured to activate if a signal generated at the electrode indicates an increase in glucose concentration with time over a predetermined rate.

42. The electrochemical sensor of claim 38, wherein at least one of the alarms is configured to activate if a signal generated at the electrode indicates a changing glucose concentration that accelerates over time above a predetermined acceleration.

43. The electrochemical sensor of claim 38, wherein at least one of the alarms is configured to activate if a signal generated at the electrode indicates a blood glucose concentration below a predetermined concentration.

44. The electrochemical sensor of claim 38, wherein at least one of the alarms is configured to activate if a signal generated at the electrode indicates a decrease in glucose concentration with time over a predetermined rate.

US 6,284,478 B1

19

20

45. The electrochemical sensor of claim 38, wherein at least one of the alarms is configured to activate if a signal generated at the electrode indicates hypoglycemia or impending hypoglycemia.

46. The electrochemical sensor of claim 38, wherein at least one of the alarms is configured to activate if a signal generated at the electrode indicates hyperglycemia or impending hyperglycemia.

47. The electrochemical sensor of claim 38, wherein the sensor of the system comprises two or more non-corroding metal or carbon electrodes and one or more alarms each coupled to two or more of the non-corroding metal or carbon electrodes.

48. The electrochemical sensor of claim 47, wherein at least one of the alarms is configured to activate if any two of the non-corroding metal or carbon electrodes to which the alarm is coupled generate electrical signals that differ by greater a predetermined amount for a predetermined period of time.

49. The electrochemical sensor of claim 47, wherein at least one of the alarms is configured to activate if any two of the non-corroding metal or carbon electrodes to which the alarm is coupled generate electrical signals that differ by greater than about 10% for a predetermined period of time.

50. The electrochemical sensor of claim 47, wherein at least one of the alarms is configured to activate if any two of the non-corroding metal or carbon electrodes to which the alarm is coupled generate electrical signals that differ by greater than about one standard deviation for a predetermined period of time.

51. The electrochemical sensor of claim 48, wherein the predetermined period of time is greater than about 10 minutes.

52. A method of calibrating an electrochemical sensor, comprising the steps of:

withdrawing a single calibration sample from an animal;

assaying an analyte concentration of the single calibration sample; and

correlating the assayed analyte concentration to a signal generated by one or more implanted working electrodes of an electrochemical sensor, each working electrode having an analyte-responsive enzyme disposed thereon.

53. The method of claim 52, wherein the sensor is an electrochemical sensor for measuring an analyte in an animal, comprising: one or more analyte responsive electrodes, at least one of said analyte-responsive electrodes adapted for subcutaneous implantation in an animal, each of the analyte responsive electrodes comprising

a non-corroding metal or carbon electrode, and

a sensing layer covering at least a portion of each non-corroding metal or carbon electrode, comprising a redox enzyme and a redox compound;

wherein the redox enzyme and redox compound are non-leachable by fluids in the body of the animal at a pH of between about 6.5 and about 7.8.

54. A method of measuring the concentration of a biochemical in an animal comprising:

contacting body fluid of the animal with the electrochemical sensor of claim 9 to generate an electrical signal; and

determining from the generated electrical signal the concentration of a biochemical in the body fluid.

55. The method of claim 54, wherein the body fluid is blood, plasma, or serum.

56. The method of claim 54, wherein the measuring is intermittent.

57. The method of claim 54, wherein the contacting includes implanting the electrode subcutaneously in the animal.

58. The method of claim 54, further comprising placing a reference electrode or combined reference and counter electrode on or in the skin of the animal.

59. The method of claim 54, wherein the biochemical to be detected is glucose.

60. The method of claim 54, wherein the biochemical to be detected is lactate.

61. The method of claim 54, wherein the sensor is capable of being calibrated by the method of claim 52.

62. The method of claim 54, wherein the sensor has two or more non-corroding metal or carbon electrodes and substantially simultaneous readings of the two or more non-corroding metal or carbon electrodes are accepted as correctly measuring the concentration of the biochemical when the two or more readings do not differ by more than a specified percentage of the measured electrical signals.

63. The method of claim 56, wherein the sensor has two or more non-corroding metal or carbon electrodes and readings of the two or more non-corroding metal or carbon electrodes that do not differ by more than about 20% or by more than about one standard deviation are accepted as correctly measuring the concentration of the biochemical.

64. A method for the analysis of a bioanalyte, comprising:

providing an analyte measurement system comprising two or more subcutaneously implantable electrodes;

subcutaneously implanting two or more electrodes in the body of an animal;

obtaining readings from each of the electrodes at substantially one point in time;

comparing two or more of the readings from the electrodes; and

accepting those readings which do not vary by more than a predetermined degree.

65. The method of claim 64, wherein the analyte measurement system is an analyte measurement system comprising:

an electrochemical sensor including two or more non-corroding metal or carbon electrodes, each electrode adapted for subcutaneous implantation in an animal, and a non-leachable analyte-responsive enzyme disposed on each of the electrodes; and a device for comparing signals generated at the two or more electrodes.

66. The method of claim 64, wherein the readings are obtained continuously.

67. The method of claim 64, wherein the readings are obtained intermittently.

68. The method of claim 64, wherein the method further comprises

measuring a temperature near one or more electrodes at substantially the same point in time as obtaining the readings from the electrodes, and

correcting the readings from the electrodes based on the measured temperature.

69. The method of claim 64, wherein the sensor is capable of calibration by a method of calibrating an electrochemical sensor, comprising the steps of:

US 6,284,478 B1

21

withdrawing a single calibration sample from an animal;

assaying an analyte concentration of the single calibration sample; and

correlating the assayed analyte concentration to a signal generated by one or more implanted working electrodes of an electrochemical sensor, each working electrode having an analyte-responsive enzyme disposed thereon.

70. The method of claim **52**, wherein a baseline current of the one or more working electrodes is 0.5 nA or less.

71. The method of claim **52**, wherein a baseline current is 1.5% or less of a total current of the one or more working electrodes at 10 mM analyte concentration.

22

72. The method of claim **52**, wherein a baseline current is 5% or less of a total current of the one or more working electrodes over a physiological analyte concentration range.

73. The method of claim **52**, wherein the one or more working electrodes are subcutaneously implanted.

74. The electrochemical sensor of claim **9**, further comprising one or more reference or reference/counter electrodes, at least one of said reference or reference/counter electrodes adapted for placement on the surface of the animal.

*   *   *   *   *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO.   : 6,284,478 B1   Page 1 of 1
DATED   : September 4, 2001
INVENTOR(S)   : Heller et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is
hereby corrected as shown below:

Title page,
Item [63], **Related U.S. Application Data**, after "Pat. No. 5,356,786" insert
-- , which is a continuation of U.S. Patent Application having Serial No. 07/664,054
filed March 4, 1991, now abandoned --.

Column 1,
Line 7, after "Pat No. 5,536,786, insert -- which is a continuation of U.S. Patent
Application having Serial No. 07/664,054 filed March 4, 1991, now abandoned --

Signed and Sealed this

Eleventh Day of February, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office